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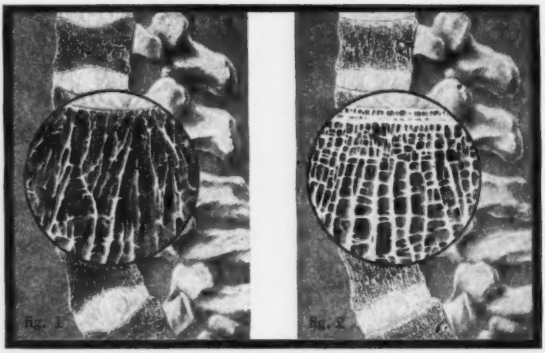
AGING CHANGES THE BONE PICTURE



lumbar vertebrae, magnified sagittal sections

The vital role that estrogen and androgen play in the preparation and recalcification of bone matrix readily explains why declining sex hormone production which accompanies aging is most frequently the cause of osteoporosis. Note typical atrophic changes characteristic of postmenopausal osteoporosis (fig. 1) in contrast to normal bone matrix (fig. 2). Reifenstein* is of the opinion that some degree of osteoporosis is almost "physiologic" after the menopause, and that clinical osteoporosis may be found in about 10 per cent of women over 50 years of age.

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*Reifenstein, E. C., Jr., in Harrison, T. R.: Principles of Internal Medicine, Philadelphia, The Blakiston Company, 1950, p. 655.

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THE RESPONSE OF THE TESTIS OF THE HAMSTER TO CHRONIC TREATMENT WITH DIFFERENT ESTROGENS[1]

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INTRODUCTION

THE atrophy which occurs in the testes of laboratory animals treated with estrogens has been well known for many years (Herrmann and Stein 1916, Laqueur and deJongh 1928, Golding and Ramirez 1928, Spencer, D'Amour and Gustavson 1932, Krohn and Zuckerman 1950, and others). Information on the reaction of the human testis is beginning to appear as there become available more cases of individuals with prostatic cancer and of criminal sexual offenders treated with estrogens (Dunn 1941). Morrione (1944), Bennett, Baggenstoss and Butt (1950) and others have described testicular atrophy in patients with hepatic cirrhosis of long standing, presumably from failure of the liver to inactivate circulating estrogens. Although there appears to be no need to review in detail the changes that have been described, briefly they include a progressive disappearance of spermatogenic elements beginning with the late stages of spermatogenesis. Ultimately the tubules appear to contain only Sertoli cells, scattered spermatogonia and an occasional spermatocyte. Interstitial cells may be unaffected (Lynch and Scott 1951), atrophy, disappear completely, or, in some strains of mice, undergo hyperplasia and malignant transformation (Gardner 1937 and 1943, Hooker, Gardner and Pfeiffer 1940, Bonser 1940, Shimkin, Grady and Andervont 1941). Lynch (1952) in the rat testis, and Dunn (1941) in the human testis have indicated that recovery from this atrophy may occur in these species after cessation of treatment. This appears also to be true in the hamster (Fig. 4).

Received August 7, 1954.

[1] The experiments from which this material was obtained were supported by grants from the American Cancer Society and the National Cancer Institute.

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MATERIALS AND METHODS

The materials upon which this report is based consist of the testes of 165 hamsters ranging in age at autopsy from 145 days to 564 days. In view of the seasonal changes shown by the hamster testis, and the infrequent occurrence of some of the alterations found in the treated animals, it was felt that a large number of controls should be studied. Therefore, of the 165 animals employed, 58 were untreated controls sacrificed at intervals throughout the year, each month being represented by at least 3 individuals.

The five compounds employed, and the number of animals studied histologically in each group were diethylstilbestrol (64), α estradiol (11), ethinyl estradiol (16), estrone (6) and Fenocylin (7 methyl bisdehydrodoisynolic acid) (10). They were administered in the form of subcutaneously implanted compressed pellets consisting of 20 mg. of the pure substance without cholesterol or other absorption retarding agents (Bacon 1950).[3] Treatment was initiated in most cases on the 50th day of age (range: 48th to 71st days).

At autopsy the left testis was removed, trimmed of adnexa, weighed, and fixed in Bouin's solution. Dehydration was carried out in ethanol and embedding in paraffin. Sections were stained with Mallory-azan or with hematoxylin and eosin and in some instances with Vehoeff's stain for elastin or Foot's silver ammonium carbonate for reticulum. Serial sections were prepared when indicated by findings in sample sections. In some instances the right testis was fixed in 10% neutral formalin for study of the distribution of lipids. These specimens were infiltrated with gelatin, cut on the freezing microtome and stained with sudan IV.

RESULTS

The gross effects of the estrogen treatment on weight of the animal, of the hypophysis and of the testis are indicated in Table 1. It is seen that the testis is reduced to about 7% of the weight in controls. The animals represented in this table were selected on the basis of approximately equivalent periods of treatment and completeness of autopsy data. For instance, in some early experiments the weight of the testis or of the hypophysis had not been recorded. Such animals were excluded from this table, but the testes were studied histologically. It should be pointed out that the body weight at autopsy may not always be significant, even though an adequate number of individuals is recorded, because of the occurrence of occasionally

TABLE 1. MALE HAMSTERS TREATED WITH VARIOUS ESTROGENS

Treatment	Age in days	Body wt., gm.	Duration of treatment, days	Pituitary wt., mg.	Wt. of left testis, mg.
Controls (9)	233	117	—	3.8	1,107
Diethylstilbestrol (10)	385	100	318	25.2	91
α Estradiol (8)	404	98	354	16.0	73
Ethinyl estradiol (15)	433	90	384	30.5	71
Estrone (6)	454	100	404	57.2	102
Fenocylin (10)	430	112	382	17.3	864

Animals in this table were selected for approximately equivalent periods of treatment and completeness of autopsy data.

[3] The pellets were supplied through the courtesy of Dr. E. Oppenheimer of Ciba Pharmaceutical Products, Inc.

massive renal tumors in animals treated for longer than 250 days with diethylstilbestrol or with α estradiol (Kirkman and Bacon 1952). In addition large amounts of ascitic fluid and/or edema were occasionally present in most treated groups.

The possibility arises that some of the testicular atrophy may be related to the debilitating effects of these tumors. That this is probably not an important factor is indicated by the occurrence of the most severe atrophy in animals treated with ethinyl estradiol, none of which developed renal tumors. Furthermore, as will be pointed out, the testis weight after 90 days of treatment had been reduced to a level where further treatment had no effect, while the earliest renal tumor to appear developed after 180 days (Kirkman and Bacon 1952).

Table 2 indicates that there was some variation in the average amount of the different estrogens absorbed as determined by weight of the pellets

TABLE 2. RATE OF ABSORPTION OF ESTROGENS FROM SUBCUTANEOUS PELLETS IN HAMSTERS

Treatment	No. animals	Duration of treatment (days)	Mg./day
Diethylstilbestrol	77	338	.09
α Estradiol	8	355	.07
Ethinyl estradiol	12	345	.17
Fenocylin	8	282	.16

The testes of some of these animals have not been studied histologically.

recovered at autopsy, desiccated, and compared with the weight at the time of implantation. This can not be accounted for by differences in the average duration of treatment, and therefore reflects differences in the rates at which the various estrogens were absorbed. An average absorption rate of 0.09 mg. per day has been calculated for diethylstilbestrol. Apparently ethinyl estradiol and Fenocylin are absorbed somewhat more rapidly and α estradiol more slowly than diethylstilbestrol.

Untreated controls showed no abnormal or unexpected structural or tinctorial characteristics. Seasonal variations occurred in the weight of the testis, diameter of the tubules, and degree of spermatogenic activity (Figs. 1 and 2), but in no instance did these testes display any but the characteristics of a normal mammalian male gonad. One testis (Fig. 3) obtained in January and showing the most extreme seasonal (perhaps pathological) reduction in activity weighed only 280 mg., and resembled in some respects the gonads of males treated with small amounts of diethylstilbestrol in pellets of cholesterol. However, even this testis was more nearly normal than those of the treated animals to be described. Similarly the testes of untreated senile animals showed features resembling those of old individuals of other species but distinct from those to be found in hamsters treated with estrogens.

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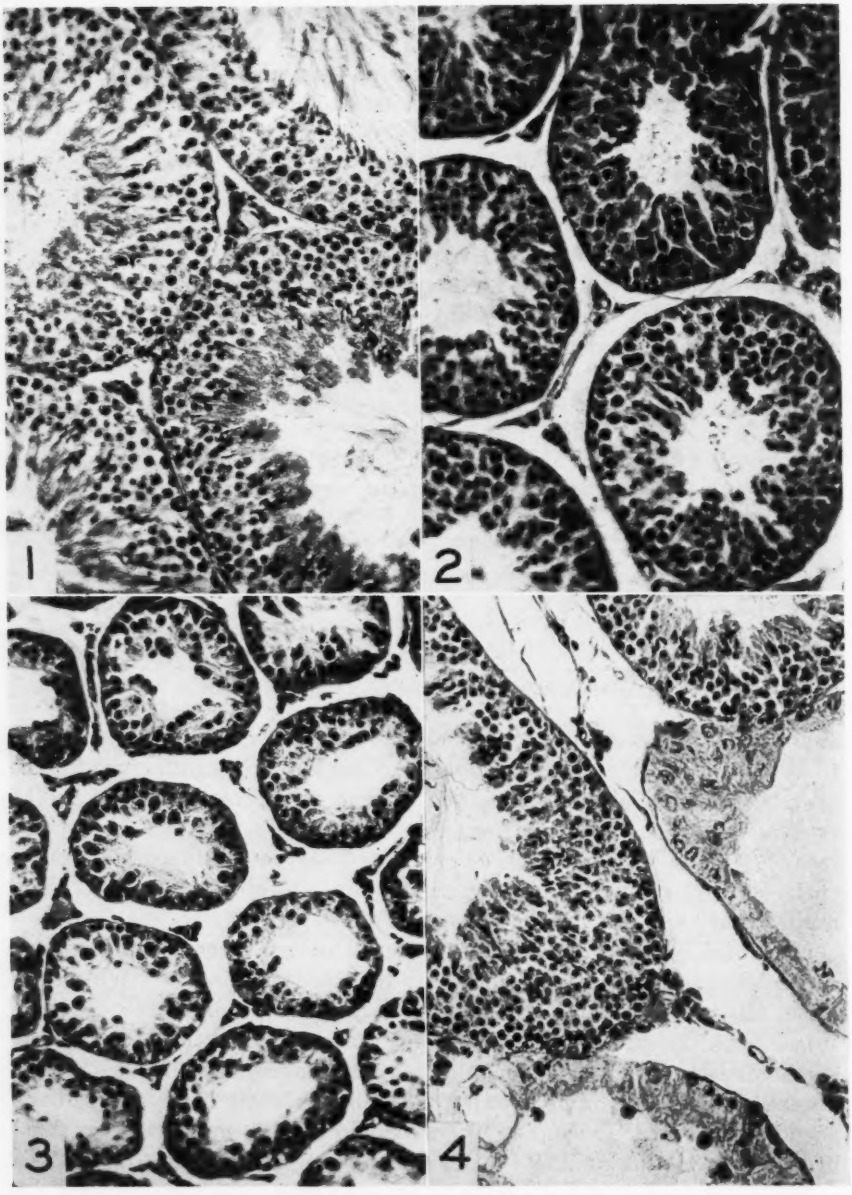

FIG. 1. Section of testis of untreated hamster autopsied in May. H. and E. ×200. Weight of testis 1863 mg.

FIG. 2. Section of testis of untreated hamster autopsied in January. H. and E. ×200. Weight of testis 700 mg. This represents the greatest activity in testes obtained during winter months.

The seminiferous tubules

General atrophic changes. Diethylstilbestrol, α estradiol and ethinyl estradiol had already brought about severe testicular atrophy in animals treated for the shortest period in this series (90 days), but Fenocylin had no effect on the histology of the tubules. Longer periods of treatment did not reduce testis weight significantly, but led to unexpected histological modifications in the remaining tissue. The material obtained from animals treated with estrone is not in most cases comparable with that from other groups because only one of these animals was in good health at the time of autopsy. The other five were described in autopsy notes as either weak and emaciated or moribund. Thus, although the histological picture of atrophy in these testes did not differ from that in other groups, part of the picture may have been due to other than endocrine factors.

All specimens showed extreme reduction in number, and in some cases nearly complete disappearance of spermatogenic elements. In general the atrophic changes followed one of two courses. The most frequently occurring sequence was a reduction in tubular diameter, first to tubules of Sertoli cells with scattered spermatogonia and primary spermatocytes (Fig. 5), and then in some instances to cords of Sertoli cells without visible lumina (Fig. 6). Testes proceeding to this degree of atrophy showed striking resemblance to fetal testes. Apparently there was some variation in the rate at which these changes proceeded. Figure 5 is of a section of testis from an animal which had received diethylstilbestrol for 151 days, while Figure 6, in which atrophy appears to have progressed somewhat farther, is of a section of testis from an animal which had been treated for 120 days.

The alternative course of atrophy, following disappearance of most spermatogenic cells, was toward the formation of cystic tubules of Sertoli cells. In more advanced stages of this condition, the epithelium became cuboidal (Figs. 7 and 8) and ultimately squamous (Fig. 9).

For convenience of discussion we have come to use the terms "solid atrophy" and "cystic atrophy" for these two degenerative pathways. Occasionally one portion of a testis contained tubules which had undergone solid atrophy, and another portion of the same testis showed cystic

FIG. 3. Section of testis of untreated hamster autopsied in January. H. and E. ×200. Weight of testis 280 mg. This illustrates the greatest reduction in activity in testes obtained during winter months. This may be abnormal, but is shown to suggest basic differences between seasonal reduction and atrophy induced by estrogens. Compare with Figure 5.

FIG. 4. Section of testis of hamster treated with diethylstilbestrol for approximately 200 days and autopsied an estimated 45 days after the last of the pellet was absorbed. H. and E. ×200. Most tubules had returned to normal, but some showed no trace of recovery.

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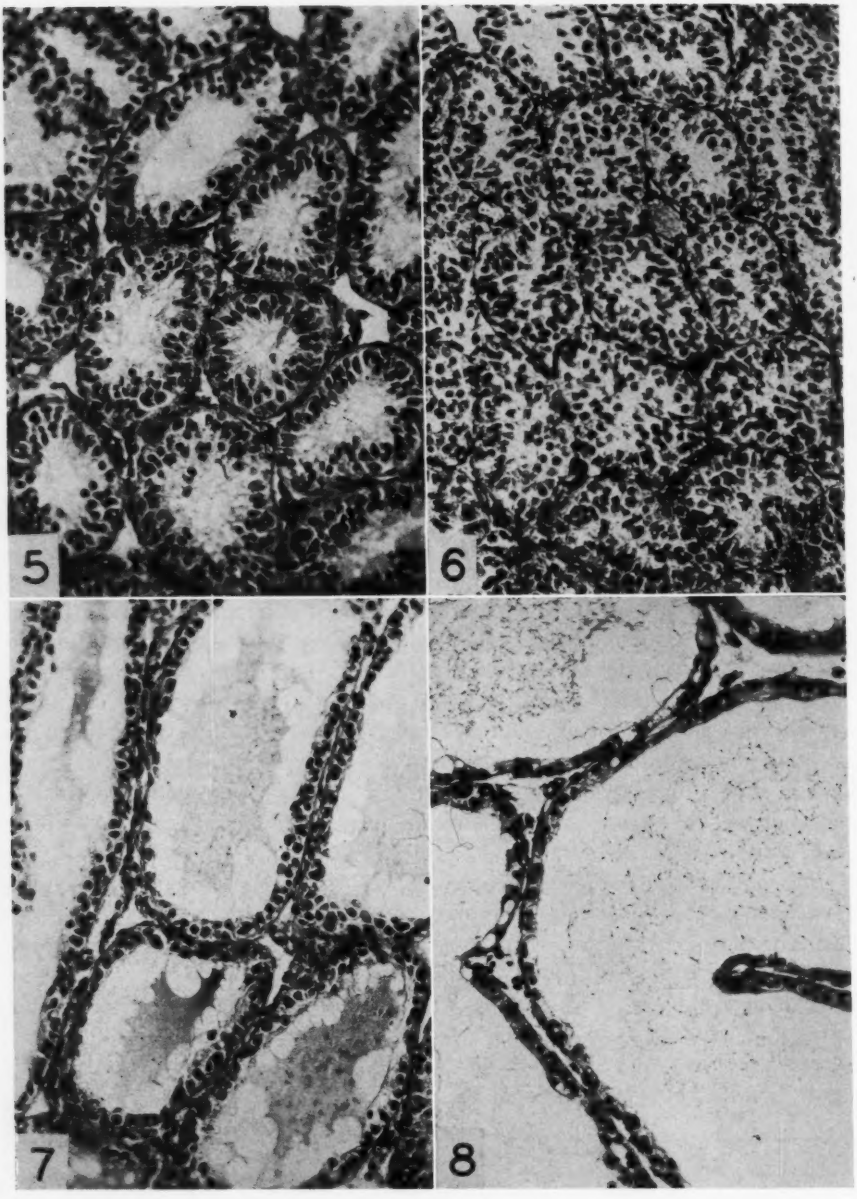

FIG. 5. Section of testis of hamster treated with diethylstilbestrol for 151 days. H. and E. ×200. Weight of testis 70 mg.

FIG. 6. Section of testis of hamster treated with diethylstilbestrol for 120 days. H. and E. ×200. Weight of testis 80 mg.

FIG. 7. Section of testis of hamster treated with diethylstilbestrol for 120 days. H. and E. ×200.

FIG. 8. Section of testis of hamster treated with diethylstilbestrol for 189 days. H. and E. ×200. Weight of testis 505 mg. Nearly all of this testis was composed of tubules of cuboidal epithelium.

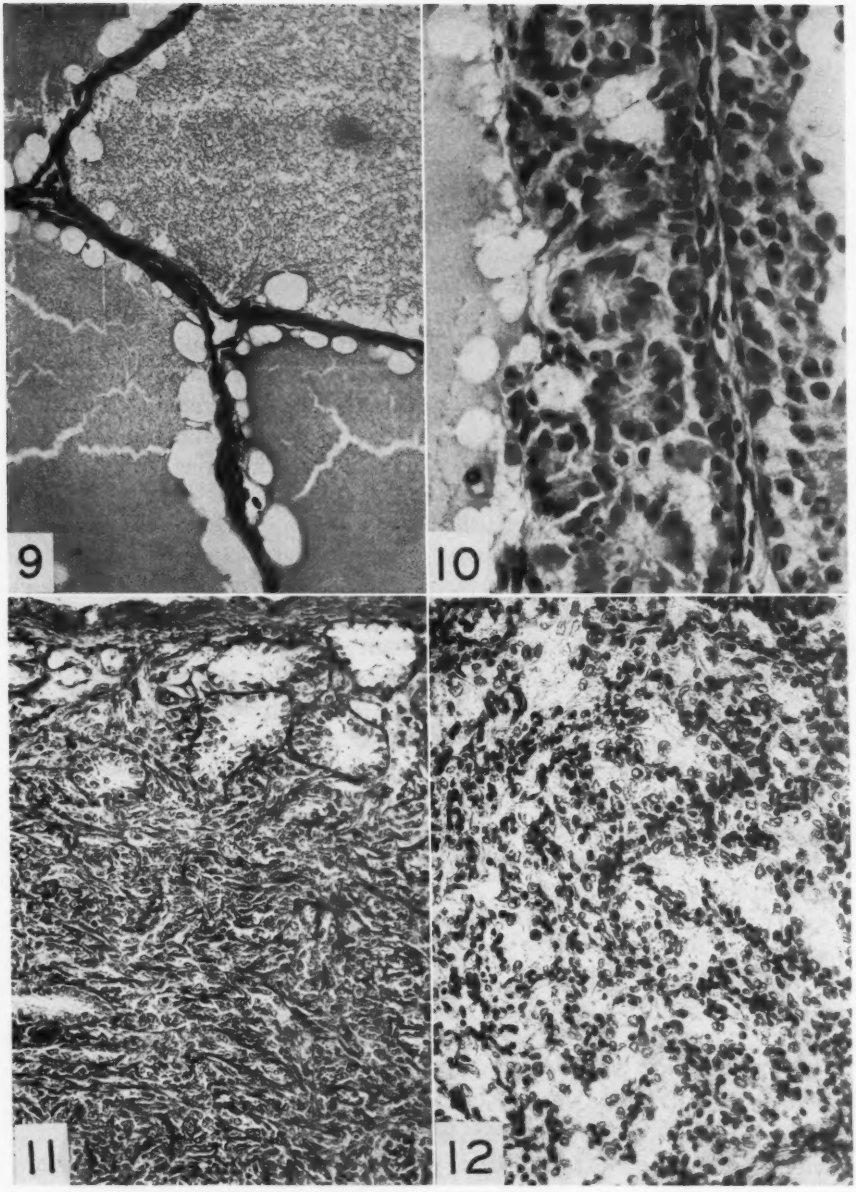

FIG. 9. Section of testis of hamster treated with diethylstilbestrol for 496 days. H. and E. ×200. Weight of testis 78 mg. Note reduction of Sertoli cell tubules to squamous epithelium.

FIG. 10. Section of testis of hamster receiving a single pellet of diethylstilbestrol and autopsied 559 days after implantation. H. and E. ×300. Weight of testis 180 mg. Note recovery in tubule adjacent to cyst.

FIG. 11. Section of testis of hamster treated with ethinyl estradiol for 400 days. Mallory. ×150. Weight of testis 139 mg. Severe atrophy with some tubular structures remaining beneath capsule.

FIG. 12. Section of testis of hamster treated with diethylstilbestrol for 450 days. Mallory. ×200. Radical breakdown of testicular architecture.

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atrophy. Characteristically these changes were accompanied by the accumulation of large amounts of sudanophilic lipid in the cytoplasm of Sertoli cells and in the lumina of the atrophic tubules. This occurred in the stages progressing toward solid atrophy, but did not appear in the cystic tubules.

As a special case of cystic atrophy, testes from 5 animals treated with diethylstilbestrol and 2 treated with ethinylestradiol contained large cysts which represented greatly dilated seminiferous tubules. The lumen of such a cyst usually contained homogenous eosinophilic material, and its walls were composed of flattened cells derived from the spermatogenic epithelium. Presumably these were Sertoli cells. In 2 cases the Sertoli cells lining such a cyst had become reorganized into small secondary tubules or cords lying within the cystic primary tubule. These secondary tubules were without a basement membrane and lacked any connective tissue support (Fig. 10).

As a special case of solid atrophy, 10 testes proceeded to a condition in which the basic tubular architecture appeared to have broken down (Figs. 11 and 12). Two entire testes showed this condition, one from an animal treated for 425 days with diethylstilbestrol, another from an animal treated for 400 days with ethinyl estradiol. Portions of 8 others, all of which were from animals treated for 400 days or longer, showed this architectural breakdown. Primarily tubular or cord-like structures resembling even the fetal testis were no longer recognizable except in the area under the capsule in the specimen illustrated in Figure 11. There was no replacement fibrosis with removal of parenchyma, but rather a disorganization of structure into irregular masses of epithelioid cells and connective tissue. The only indication of formed structures in such areas was seen in the occasional presence of rosette-like clusters of cells arranged about small cores of connective tissue fibers. These cells appeared to be modified Sertoli cells. There were some areas of intense mitotic activity in one of these testes which may represent clusters of germinal elements scattered through the reorganized tissue.

Rosette formation. Following the disappearance of spermatogenic cells, seminiferous tubules in 5 testes underwent local change in structure with the formation of a mass of radially arranged cell clusters filling the lumen. In these cases the tubules were locally much enlarged and the lumen obliterated by the clusters. The most advanced development of this arrangement is shown in Figure 13 where each cluster may be seen to consist of a core of connective tissue covered by a layer of Sertoli cells and a few spermatocytes. Study of serial sections indicated that this change was confined to a single tubule. In cross section these structures resembled closely the rosette-like disposition of cells and connective tissue already mentioned as being found in some areas of testes which have undergone extensive architectural breakdown. This transformation, occurring locally

in a single tubule, may be an early stage in the initiation of the more radical change affecting the entire organ.

Inflammatory reactions. In many testes the presence of varying degrees of chronic inflammation was suggested by masses of lymphoid cells or of larger phagocytic elements usually containing lipids. This condition was most frequently encountered in the lower portions of the seminiferous tubules, in the tubuli recti, and in the rete testis (Fig. 14). Neutrophilic infiltration was encountered only in the epididymis and other accessory organs where abscesses of astonishing size occasionally appeared. Scattered intratubular phagocytic cells which were common in all treated groups, were demonstrated by subcutaneous administration of trypan blue several days before autopsy.

The rete testis and ductuli efferentes

The rete and efferent ductules showed extensive hyperplastic and metaplastic alterations in 6 animals which had received diethylstilbestrol and 3 which had received ethinyl estradiol. The epithelium of the rete became tall, intensely staining columnar or pseudostratified columnar, with scattered intraepithelial spaces containing basophilic material. Similar but less severe changes occurred in 2 estradiol treated animals. In three instances there was extensive local proliferation of basal cells transforming the epithelium to a stratified columnar type (Fig. 15). One of these cases had a massive hyperplasia of these basal cells.

Large cystic dilatations of the rete or efferent ductules were common and usually were associated with cystic seminiferous tubules within the testis proper (Fig. 16). This association may be related to disturbances in the absorption of fluid which is said to occur in the epididymis (Mason and Shaver 1952).

The interstitial tissue

In most specimens cells of Leydig could not be identified. When found they were small, sometimes had pyknotic nuclei, but more frequently resembled connective tissue cells as reported by Koneff, Simpson and Evans (1946) following treatment of hamsters with diethylstilbestrol for shorter periods. Although Fenocylin had essentially no effects on the seminiferous tubules or on weight of the testis, there was considerable reduction in number, size, and intensity of staining of Leydig cells in 8 of the 10 animals of this group. However, since most of the animals treated with this substance were autopsied during the winter months, this may be a seasonal effect.

Many testes, except those of animals receiving Fenocylin, showed indications of an increase in the number of cells in the interstitial spaces. This reached its greatest development in animals treated with estrone, in which it appeared that the hyperplasia was sufficient, even with extreme tubular atrophy, to account for the slightly higher average weight of the testes in

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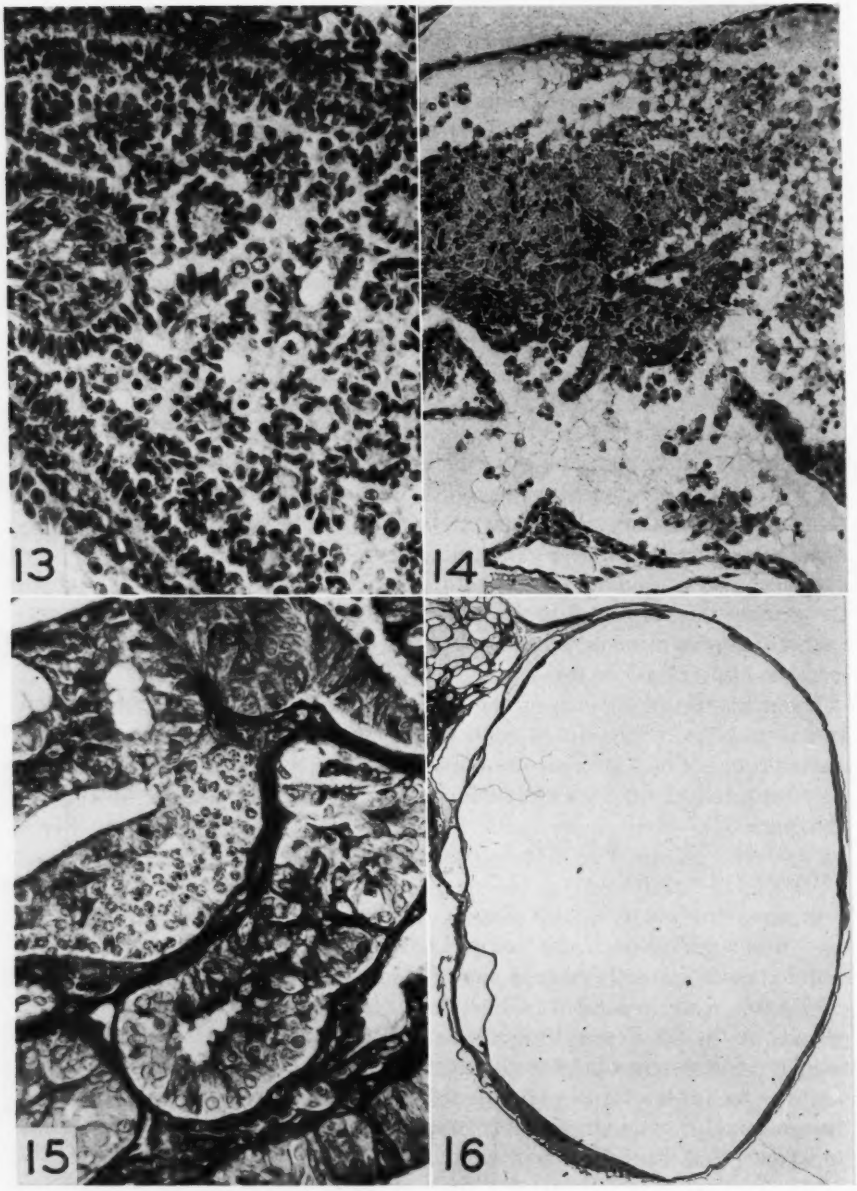


FIG. 13. Section of testis of hamster treated with diethylstilbestrol for 327 days. H. and E. ×300. Weight of testis 80 mg. Narrowed portion of a distended seminiferous tubule containing clusters of radially arranged cells around connective tissue centers. A few germinal cells and several mitotic figures may be seen within this tubule.

this group. When compared with fibroblasts in the connective tissue around blood vessels in the same sections, these cells were seen to possess smaller, more nearly spherical nuclei with more intensely staining nuclear membranes and much coarser chromatin granules. They gave the impression of being intermediate in structure between fibroblasts and Leydig cells.

Staining by Verhoeff's method for elastin gave no indication of any change in elastic fibers or membranes in estrogen induced atrophy.

Nearly all testes, except those from animals treated with Fenocylin, contained small local accumulations of macrophages, usually associated with what appeared to be the final stages in the removal of atrophic tubules and Leydig cells. They usually contained brown or yellow pigment in varying amounts.

New structures of uncertain origin

In addition to histological alterations clearly assignable to either seminiferous tubules or the interstitial tissue, certain new features appeared whose relation to normal testicular morphology was not apparent.

Epithelial tubules. Tubular structures in no way resembling even the simplest tubules of Sertoli cells and never containing any spermatogenic cells occurred in the testes of 18 animals treated with diethylstilbestrol and of one animal treated with α-estradiol. They were usually of simple columnar epithelium with basally located spherical (less frequently oval) nuclei, and quite dense acidophilic cytoplasm. In most cases the cells were of remarkably uniform height and width, with sharply outlined cell boundaries, terminal bars (Fig. 19), and a zone of condensed darkly staining cytoplasm at the free surface. In one instance cilia could be demonstrated (Fig. 20).

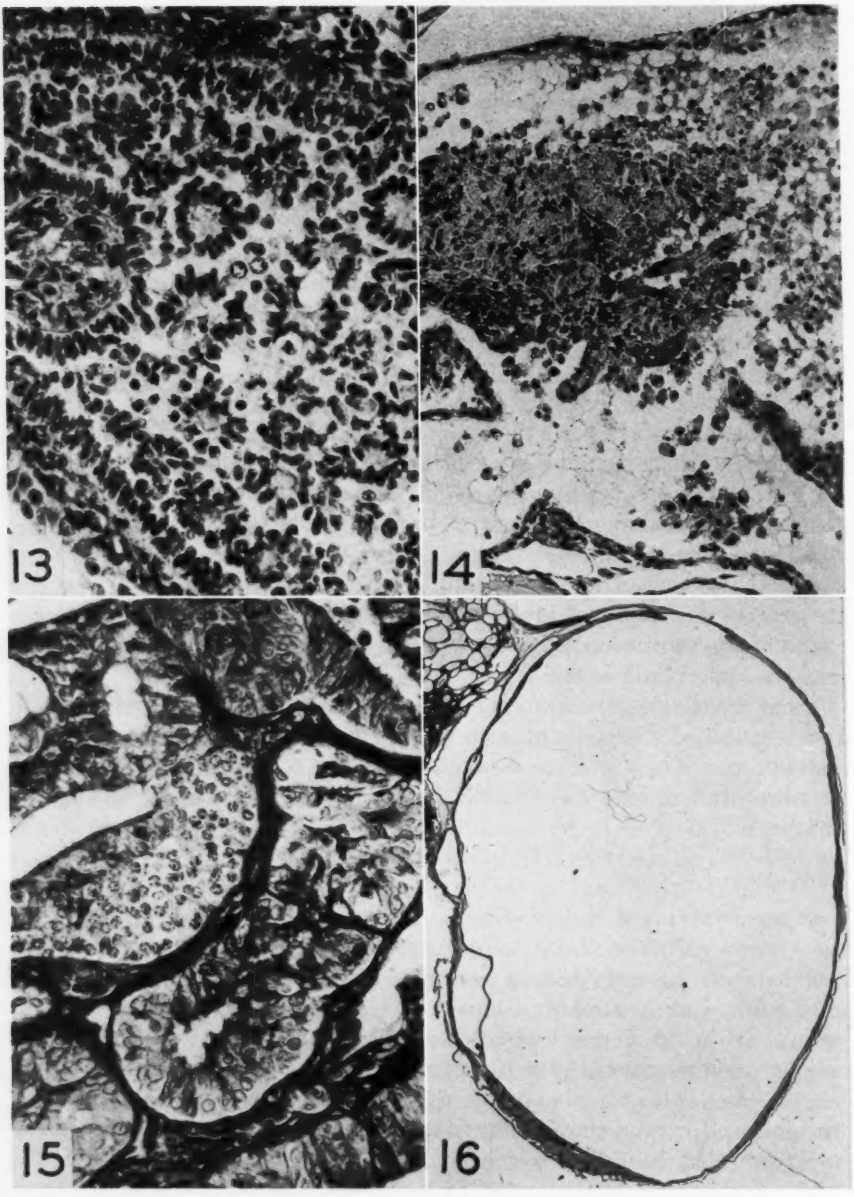
In addition to these tubules, large cysts were encountered lined with cuboidal or tall columnar cells with basophilic granular cytoplasm seeming to represent an actively secreting epithelium, perhaps derived from the epithelial tubules just described.

Tumors. In three specimens tumors appeared. In two cases these were adenomas; the third appeared to be an early Sertoli cell tumor or possibly more simply an excessive Sertoli cell hyperplasia. The first to be described may have been of multiple origin since the mass shown in Figure 21 did

FIG. 14. Section of testis of hamster treated with diethylstilbestrol for 330 days. H. and E. ×150. Masses of phagocytic elements occupy the channels of the rete.

FIG. 15. Section of rete testis at junction with ductuli efferentes from hamster treated with ethinyl estradiol for 400 days. Mallory. ×300. Hyperplastic pseudostratified and stratified columnar epithelium.

FIG. 16. Section of cyst of ductus efferens from hamster treated with diethylstilbestrol for 496 days. H. and E. ×10. Note that many seminiferous tubules in the testis proper are also cystic.



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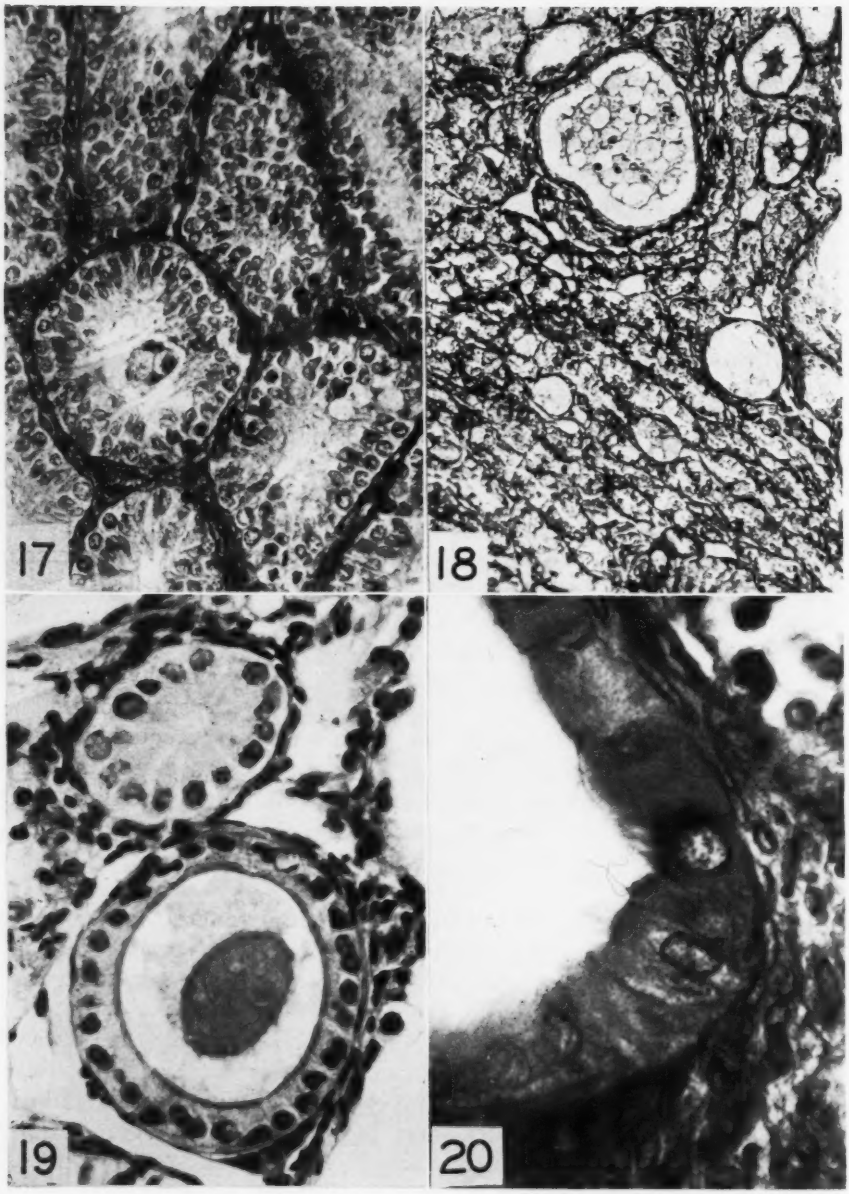

FIG. 17. Hyperplasia of cells of interstitial tissue in testis of hamster treated with ethinyl estradiol for 400 days. Mallory. ×300. Compare with Figures 4 and 5.

FIG. 18. Fibrous proliferation of interstitial tissue in testis of hamster treated with ethinyl estradiol for 400 days. Mallory. ×150.

FIG. 19. Section of epithelial tubules in testis of hamster treated with diethylstilbestrol for 410 days. H. and E. ×500. Note terminal bars and clear cell boundaries in upper tubule, and darkly staining surface cytoplasm of cells in lower tubule.

FIG. 20. Section of epithelial tubule in testis of hamster treated with diethylstilbestrol for 372 days. Iron hematoxylin. ×1000. Ciliated cells.

not appear to be in continuity with a histologically identical, and much larger mass at the mediastinal border of the testis in the region of the rete and efferent ductules. It occurred in an animal which had received pellets of diethylstilbestrol and of diethylstilbestrol in cholesterol (1:4). Treatment had extended over a period of 521 days. The tumor was composed of tall columnar cells of uniform size arranged in cords and tubules, and did not appear to be malignant. The second case (Fig. 22) was a much larger mass weighing 6777 mg. located at the mediastinal border of the left testis (126 mg.) in an animal which had received ethinyl estradiol for 400 days. It was essentially of the same structure as the preceding tumor but less densely arranged and with some areas at the periphery which were suggestive of invasiveness. The testis proper showed no evidence of tumor tissue, a fact which suggests that in the previous instance the intratesticular mass may have extended into the parenchyma of the gonad from an outside origin.

In one testis, from an animal treated with α estradiol for 514 days, excessive hyperplasia of Sertoli cells amounting to a tumor greatly distended one seminiferous tubule (Fig. 23). The basement membrane of this tubule appeared to be still intact and may have served to limit the extension of the growth. This was suggested by the presence of large necrotic masses at other levels of the same much distended tubule.

Four animals treated with ethinyl estradiol showed epithelial hyperplasia in the rete or ductuli efferentes, three of which could with some justification be classed as tumors. The fourth was a small area of hyperplastic epithelium histologically identical with the other three, and therefore was considered as a possible early stage in tumor development.

Vascular lesions

Vascular lesions were noted in the testes of 4 animals which had received diethylstilbestrol and one which had received α estradiol (Fig. 24). These consisted of intimal thickenings with deposits of hyalin (demonstrated with Mallory's phloxine method). These lesions were limited to vessels in the center of the testis and occurred in a group in which treatment with diethylstilbestrol had been discontinued to study the recovery process. Since these animals averaged only 380 days of age at autopsy, and since no such lesions were found in any other animals of any group treated with diethylstilbestrol alone, or in any untreated animal even in advanced senility, these do not appear to be arteriosclerotic age changes.

DISCUSSION

In discussing the effects of the four estrogens employed, Fenocylin may be eliminated immediately as having essentially no effect on the hamster testis. Although it reduces testicular weight somewhat (the average is still within the range of control weights, however) and brings about striking

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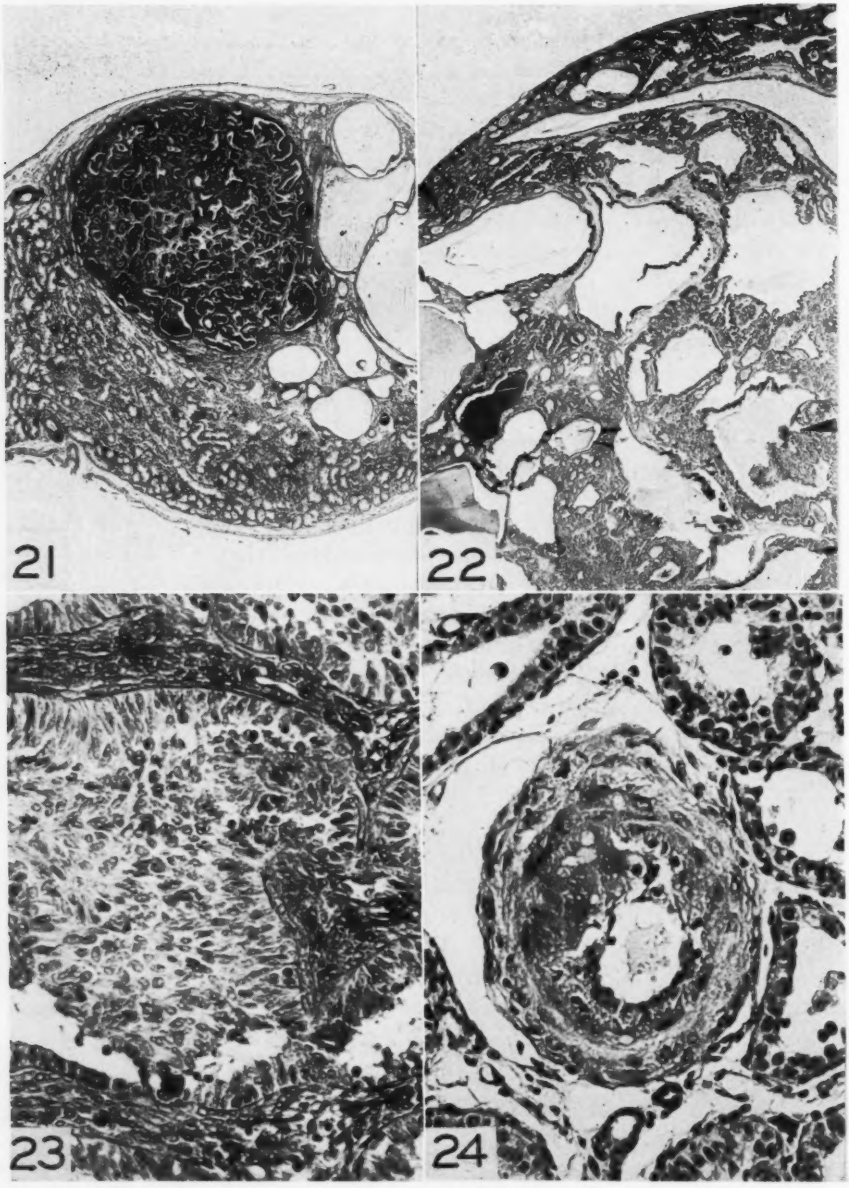

FIG. 21. Section of testis of hamster receiving pellets of diethylstilbestrol and diethylstilbestrol in cholesterol for 521 days. Mallory. ×15.

FIG. 22. Section of large mass removed from mediastinal border of testis of hamster treated with ethinyl estradiol for 400 days. H. and E. ×7.5. There was no extension of the tumor into the testis. Weight of tumor 6777 mg.; weight of testis 126 mg.

changes in some of the accessory glands and in the hypophysis, it has no demonstrable effect on tubular morphology. There may be some suppression of Leydig cells, but on the basis of a study of the testis alone, this substance probably would not be classified as an estrogen.

Ethinyl estradiol appears to bring about the most drastic changes in the histological structure of the testis, with α estradiol and diethylstilbestrol bringing about less damage but being approximately equivalent in their effects. It is apparent from Table 2 that it would be difficult to assign other than a quantitative basis for this difference since the absorption rate of ethinyl estradiol was approximately twice that of α estradiol and of diethylstilbestrol.

Vasquez-Lopez (1944) listed in a table two cases of carcinoma of the caput epididymis in estrogen treated hamsters. Whether these are similar to the tumors we have described above as adenomas is uncertain since his tumors were not described or illustrated. In our material there appear to be three possible sources of the tumors: (1) the aberrant epithelial tubules of unknown origin described above, (2) Sertoli cells, (3) epithelium of the rete, ductuli efferentes or caput epididymis. Although the cells are in some respects similar, the first possibility seems unlikely since aberrant tubules did not occur in those animals treated with ethinyl estradiol. Stalker and Hendry (1952) have recently reviewed and discussed the rare "tubular adenoma" of the human testis and feel that this tumor is of Sertoli cell origin. The hamster tumors resulting from treatment with ethinyl estradiol or diethylstilbestrol do show some resemblance to the human tubular adenoma, but we feel that the origin of our tumors from Sertoli cells is unlikely. The clearest case of Sertoli cell hyperplasia occurred in an animal treated with α estradiol and these cells showed no resemblance to those of the adenomas. The third possibility seems to be the probable source of our tumors. Epithelial hyperplasias of the ductuli efferentes and rete were common and were most extensive in animals receiving ethinyl estradiol. Indeed, in this group they were sometimes classified as early stages in tumor development. Similar hyperplasias occurred less commonly following treatment with diethylstilbestrol and infrequently in α estradiol treated animals. In addition, a large extratesticular tumor, identical to that within the gonad was found in the case treated with diethylstilbestrol,

FIG. 23. Section of testis of hamster treated for 514 days with α estradiol. Mallory. $\times$250. Note area of Sertoli cell hyperplasia. Other areas of greater extent were present in this tubule, but this field demonstrates most clearly the relation of hyperplastic tissue to wall of seminiferous tubule.

FIG. 24. Section of testis of hamster receiving one pellet of diethylstilbestrol and autopsied 331 days after implantation. H. and E. $\times$250. Note general thickening of intima of arteriole with considerable local deposition of eosinophilic material on one side.

and only an extratesticular mass was found in the animal treated with ethinyl estradiol.

The frequent cellular hyperplasia of the interstitial tissue is of interest in connection with the reports of Hooker and Pfeiffer (1942) and of Montagna and Hamilton (1951). The former authors indicated that, in estrogen-treated mice, the Leydig cells disappeared and the interstitial cell tumors arose from a new generation of interstitial cells derived from connective tissue or mesenchymal cells. Montagna and Hamilton have described in the human testis fibroblast-like cells containing Schultz positive, autofluorescent, and sometimes birefringent lipids, which might be precursors or stages in a functional cycle of Leydig cells. The cells involved in the mild hyperplasia we have seen in the hamster appear to differ in some respects from Leydig cells, but do not entirely resemble fibroblasts. They suggest very strongly an intermediate condition between the two.

Some of the changes which appeared in the atrophic testes under continued treatment with estrogens suggest, but of course do not unequivocally demonstrate, some degree of action of these substances directly upon the testis rather than entirely by way of hypophyseal inhibition. The uniformly occurring severe tubular and Leydig cell atrophy fit well into the picture of gonadotrophin deprivation, but the case of Sertoli cell hyperplasia, the proliferation of rosettes within tubules, the development of epithelial tubules not found in testes of untreated animals, the adenomas (these, however, may be of extratesticular origin), and the epithelial cysts suggest a direct stimulating effect of some sort.

Although in the human testis Nelson (personal communication) has found distinctly increased formation of elastic fibers in the peritubular tissue in many conditions where spermatogenesis is declining, including estrogen induced atrophy, he has not seen such clear cut effects in other forms. In our material no noticeable change in elastin content, as indicated by Verhoeff's method, appeared after estrogen treatment.

The vascular lesions encountered in this material are of interest in view of the reports of Lindsay *et al.* (1946), Chaikoff *et al.* (1948) and Horlick and Katz (1948) that sustained hyperlipemia and atheromatosis occur in chickens which have received diethylstilbestrol by means of implanted pellets. However, their reported descriptions are limited to changes in the aorta, hence it is not possible to compare the morphology of their lesions with those of the testicular arterioles. More frequent and more severe lesions occur in testicular vessels of other groups of hamsters treated with diethylstilbestrol; these will be reported elsewhere.

SUMMARY

Changes in weight and histological structure of the hamster testis have been studied following chronic treatment with pellets of diethylstilbestrol, α estradiol, ethinyl estradiol, Fenocylin, or estrone.

All estrogens brought about great hypophyseal hypertrophy and all but Fenocylin resulted in reduction of testis weight to about 7% of the average weight in controls.

Fenocylin had essentially no effect on testicular morphology, but the other estrogens brought about extreme tubular atrophy and disappearance of Leydig cells. Other histological changes observed were disorganization or breakdown of testicular architecture with rosette formation, cellular hyperplasia of interstitial tissue, adenomas (probably of extratesticular origin), epithelial tubules and cysts of unknown origin, hyperplasia, metaplasia and cysts of rete and ductuli efferentes, and arteriolar sclerosis.

It is suggested that some of the changes indicate the possibility of some degree of direct action of the estrogens upon the gonad rather than an entirely indirect effect by inhibition of the hypothysis.

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A METHOD FOR THE STUDY OF THE DIABETOGENIC ACTIVITY OF PITUITARY GROWTH HORMONE PREPARATIONS IN THE RAT

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STUDIES on the nature of the diabetogenic actions of pituitary growth hormone have been impeded by the paucity of suitable techniques for the detection and quantitative estimate of this activity. The dog and cat are the species most sensitive to the diabetogenic action of growth and an assay procedure using the cat has been devised by Reid (1952, 1953). While this technique has been used most effectively by Reid it does suffer from the disadvantage that the cat is a relatively expensive animal to keep in the laboratory.

It would obviously be a considerable advantage if one of the smaller, more standard laboratory animals, such as the rat or mouse could be adapted for test purposes. Unfortunately, both these species are relatively insensitive to the diabetogenic action of growth hormone unless previously made susceptible by partial pancreatectomy (Long, Katzin and Fry, 1940) or by alloxan treatment (Milman and Russell, 1950) or as in the case of the mouse when there is a congenital predisposition to diabetes (Mayer and Silides, 1953). None of these procedures present any great advantages over the use of the cat or dog.

It has been demonstrated in this laboratory that the intact rat, force fed a high carbohydrate diet, may develop diabetes if treated simultaneously with growth hormone and a subdiabetogenic dose of ACTH (Engel, Viau, Coggins and Lynn, 1952). The present study represents an attempt to exploit the above observation for the development of a technique for the detection and estimation of the diabetogenic potentialities of pituitary extracts. By substituting cortisone acetate for ACTH as the sensitizing agent a reasonably reliable screening method has finally been developed, but it has not yet been possible to refine it to the degree necessary to meet the exacting requirements of a bioassay. Nevertheless, since there is at present a wide interest in the biological activities of pituitary growth hormone preparations we feel justified in presenting the method at this

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time so that it might be applied by other investigators and, perhaps, modified to make it acceptable as a quantitative assay.

METHODS

Male albino rats of the Vanderbilt strain, weighing 270–320 gms. at the start of the experiment were used. The animals were kept in metabolism cages in an air-conditioned room at a temperature of 28–29° C. The urine was collected with thymol or sodium fluoride as a preservative, and analyzed for sugar by the method of Benedict (1911). Blood sugar analyses were performed according to Somogyi (1945) on tail blood obtained without anesthesia.

The animals were fed by stomach tube a high carbohydrate diet (Table 1), modified from Ingle *et al.* (1947). This diet contains 0.45 gm. of carbohydrate, 0.07 gm. of protein, 0.009 gm. of fat, 2.31 calories and 0.652 gm. of solid per milliliter.

Potassium chloride in addition to that in the salt mixture was added to ensure against potassium depletion as a consequence of the cortisone treatment or the diabetes itself. Potassium depletion has been shown to modify carbohydrate tolerance in the rat (Gardner *et al.*, 1950), particularly in relation to steroid action (Glafkides, Bennett and George, 1952; Kinsell, Balch and Michaels, 1953). During the early phases of this work a number of the tube fed rats died from respiratory infection. This was largely eliminated by the addition of the antibiotic tetracycline hydrochloride to the diet. A comparison of the response to the hormones of the rats with and without the antibiotic in the diet revealed no significant difference.

TABLE 1. COMPOSITION OF TUBE FEEDING DIET

Cellu Flour or Alphacel (Nutritional Biochemical Corp.)		120 gm.
Osborne-Mendel Salt Mixture, Wesson Modification (Nutritional Biochemical Corp)		40 gm.
Calcium carbonate	21.000%	
Copper sulfate	0.039%	
Ferric phosphate	1.470%	
Manganous sulfate	0.020%	
Magnesium sulfate	9.000%	
Potassium aluminum sulfate	0.009%	
Potassium chloride	12.000%	
Potassium dihydrogen phosphate	31.000%	
Potassium iodide	0.005%	
Sodium chloride	10.500%	
Sodium fluoride	0.057%	
Tricalcium phosphate	14.900%	
Brewers' yeast, U.S.P. (Pabst or Nutritional Biochem Corp.)		100 gm.
Corn starch, soluble, U.S.P. (Amend Drug & Chemical Co.)		330 gm.
White dextrin (Merck or Mallinckrodt		335 gm.
Dextrose, U.S.P. (Merck)		335 gm.
Egg albumin (Nutritional Biochemical Corp.)		160 gm.
KCl (Merck)		8 gm.
Mazola oil (Corn Products Refining Co.)		10 gm.
Menadione (Nutritional Biochemical Corp.)		100 mg.
Cod liver oil, U.S.P. (Nutritional Biochemic. Corp.)		10 gm.
Thiamine, U.S.P. (Mallinckrodt)		7.1 mg.
Riboflavin (Nutritional Biochemical Corp.)		14.2 mg.
Pyridoxine (Merck)		7.1 mg.
Ca Pantothenate (Merck)		42.6 mg.
Niacin, U.S.P. (Merck)		142 mg.
P-aminobenzoic acid (Nutritional Biochemical Corp.)		142 mg.
i-Inositol (Nutritional Biochemical Corp.)		426 mg.
Biotin (General Biochemicals, Inc.)		1.42 mg.
Pteroyl glutamic acid (General Biochemicals, Inc.)		2.8 mg.
Choline (Merck)		7.1 mg.
Tetracycline Hydrochloride (J. B. Roerig & Co.)		2.25 mg.

H_2O q.s., 2,250 ml.

The animals were adapted to the tube feedings over a 4- to 7-day period with gradually increasing daily amounts of diet to avoid food shock (Ingle *et al.*, 1946) until the full feeding of 5.5 ml. of diet per feeding per 100 gm. of body weight was achieved. During the period of adaptation pellets of rat chow were left in the cages. The diet was administered each day at 8:30 a.m. and 5:00 p.m.

Cortisone acetate and growth hormone were injected subcutaneously immediately after the morning feeding. Cortisone acetate was supplied by Merck and Company as saline suspensions at concentrations of 5 or 25 mg./ml. The latter was diluted to 5 mg./ml. with either saline or the aqueous vehicle No. 1 of the Merck Company. Three lots of growth hormone were used. These were Horner Company, Ltd. of Montreal, Canada, lots #C-24-34-D, C-35-39-J, prepared from beef pituitary glands by the method of Wilhelmi, Fishman and Russell (1948) and Lot #PR1 derived from hog pituitary according to Raben and Westermeyer (1951).

The growth hormone solutions were prepared immediately prior to use in amounts sufficient to last five days and were stored in the refrigerator at 4° C. Lots C-24-34-D and C-35-39-J were dissolved in 0.1 N NaOH and immediately brought to pH 9.5 with 0.1 N HCl. Lot PR1 was prepared in a variety of ways, as indicated below, in order to determine the effects of various chemical and physical procedures on its diabetogenic activity. In all instances distilled water or saline was added so that the final concentration of pituitary extract was in the range of 5–10 mg./ml. No more than 0.8 ml. of solution was ever injected at one time, the usual dose being 0.4–0.6 ml.

During the course of a study of the effects of various procedures on the biological activities of PR1, reported elsewhere (Engel, 1955), the following methods of preparation for injection were employed.

1. Saline-acetic acid, pH 3.5. The growth hormone preparation was suspended in saline and brought into solution by the dropwise addition of glacial acetic acid to pH 3.5.
2. Saline-acetic acid pH 3.5, boiled. Same as above, but the solution was placed in a vial in a boiling water bath for 30 minutes prior to use.
3. HCl-boiled. The powder was dissolved in 0.2 N HCl, heated in a boiling water bath for 30 minutes and then brought to pH 3.5 with 0.2 N NaOH.
4. Alkaline dissolved, final pH 3.5. The extract was dissolved in 0.1 NaOH and immediately brought to pH 3.5 with 0.1 N HCl.
5. Alkaline dissolved, final pH 3.5, boiled. Same as #4 but boiled for 30 minutes.
6. Alkaline dissolved, final pH 9.5. Same as #4, but brought to a final pH of 9.5.
7. Alkali treatment. Dissolved in 0.1 N NaOH, allowed to stand overnight (16–20 hours) at 4° C. Adjusted to pH 3.5 with 0.1 N HCl.

RESULTS

Standardization of tube-feeding. The first requirement of the procedure was to determine the stability of the blood sugar after full tube-feeding. Ingle *et al.* (1947) have shown that adaptation to tube-feeding is necessary before any metabolic studies can be conducted, and we have confirmed this (Engel *et al.*, 1952). Figure 1 shows that during the first 10 days after full tube-feeding the blood sugar level one hour after feeding was variable and frequently still quite elevated. After ten days it became quite stable and remained so at 1, 2 and 3 hours after feeding up to as long as 60 days of feeding. On this program, the animals grew and thrived. From these data it was concluded that the tube-fed rat could be used for this study any time after ten days of full tube-feeding.

Priming dose of cortisone acetate. A key requisite of the proposed technique was to employ a dose of cortisone acetate which would permit the diabetogenic effect of growth hormone to become apparent but which itself would not induce hyperglycemia or glycosuria. For this purpose daily doses of 10, 5 and 2.5 mg. of a saline suspension of cortisone acetate (5 mg./ml.) were injected subcutaneously into groups of 3 to 6 tube fed

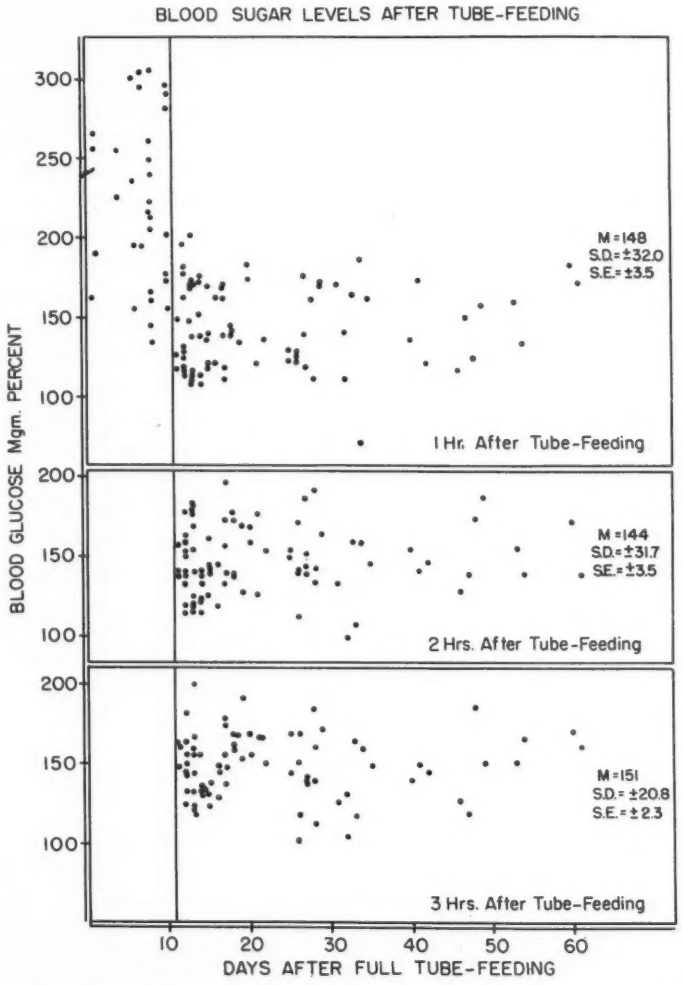

FIG. 1. Blood glucose levels of rats during 60 days of high carbohydrate tube feeding. Note that during the first 10 days of full tube-feeding the blood glucose values 1 hour after feeding are high and variable, but thereafter at 1, 2, and 3 hours *post cibum* they are quite regular. The figures on the right represent the mean blood sugar ±S.D. and S.E. after the 10th day of full tube-feeding.

rats. Body weight, sugar excretion and the blood sugar level 2 hours after feeding were measured and recorded. Representative data from this experiment are shown in Figure 2. The two larger doses of cortisone regularly caused hyperglycemia, glycosuria and weight loss, many of the rats succumbing after several days of treatment with the 10 mg. dose. With the 2.5 mg. dose of cortisone acetate no significant hyperglycemia or glycosuria occurred in 6 rats and their weights showed little change during 4–5 days of treatment. This latter dose was therefore chosen as the "subdiabetogenic" priming dose of cortisone acetate.

Ingle, Prestrud and Nezamis (1951) have reported that tube-fed rats receiving a diabetogenic dose of cortisone acetate show a diminution in glycosuria after 8–10 days of treatment and a decline from maximal liver glycogen accumulation after the fifth day (Ingle, Meeks and Beary, 1953). Our studies confirmed those of Ingle and hence the data need not be recorded in detail. Thus, after 7–10 days of treatment the diabetic response to 5 mg. of cortisone acetate daily diminished and finally disappeared. Furthermore, it was noted that after rats had received the subdiabetogenic dose of 2.5 mg. of cortisone acetate for 5–7 days, they did not exhibit hyperglycemia or glycosuria when given 5 mg. of the hormone daily. Because of these observations, which indicate some sort of a metabolic

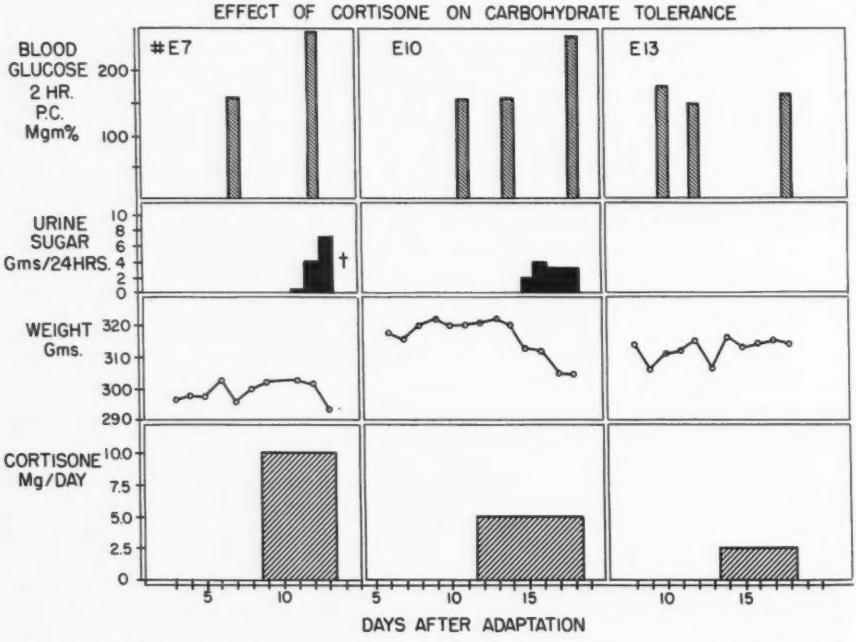

FIG. 2. The influence of variations in cortisone acetate dosage on weight, glycosuria and the two hour *post cibum* blood glucose levels of tube-fed rats.

adaptation of the rat to cortisone, it was decided to limit hormone treatment for test purposes to a 5 day period.

Previous experience with the production of diabetes by combined treatment with growth hormone and ACTH indicated that the two hormones had to be given simultaneously in order to cause diabetes (Engel *et al.*, 1952). A pilot study with growth hormone and cortisone (2.5 mg./day) showed that the same relationship held with this hormone combination. Diabetes did not develop if the hormones were administered sequentially. However, because of the limited solubility and slow release of cortisone from a subcutaneous depot (Porter and Silber, 1953), the cortisone treatment was begun 24 hours before the growth hormone.

Finally, although previous and preliminary data indicated that diabetes might develop within 24 hours of initiating treatment with the two hormones, it sometimes was delayed, particularly when less potent growth hormone preparations were employed. For this reason a 5-day period of hormone treatment was arbitrarily chosen for the final test procedure.

Standardization of the procedure for the detection of the diabetogenic activity of growth hormone. The above conditions for tube-feeding and priming with cortisone having been established, a pilot experiment was conducted with a single lot of growth hormone (C-23-34-D, Horner) in order to determine (a) whether the blood or the urine sugar was the best index of diabetes, (b) whether the blood sugar level at either 1, 2 or 3 hours had any advantage over the other hours and (c) whether there was any reasonable correlation between the dose of growth hormone and the degree of glycosuria or post prandial hyperglycemia. The procedure adopted was as follows:

1. The animals were adapted to tube-feeding, placed in metabolism cages for 24 hour urine collections and used any time after the tenth day of full feeding.

2. Except for the tube-feeding controls and the growth hormone control, 2.5 mg. of cortisone acetate were injected on the morning of days 0–4.

3. On days 1–5 growth hormone in the doses indicated in Figure 3 was injected into the appropriate animals each morning, immediately after tube-feeding.

4. On the 2 days prior to hormone treatment and on the 4th and 5th days of hormone injection blood samples obtained 1, 2 and 3 hours after the morning tube-feedings were analyzed for glucose. In the group receiving 2.2 mg. of G.H. only the 2 hour *post cibum* blood glucose levels were measured. Since subsequent inspection of the data indicated that greater uniformity was achieved when the mean values for blood sugar and glycosuria on the 4th and 5th days were pooled, only these data are recorded in Figure 3. The following groups of rats were used: 1. Control animals subjected only to tube-feeding. 2. Cortisone controls, receiving 2.5 mg. of cortisone daily during the test period. 3. Growth hormone

controls receiving 0.55 mg. G.H./100 gm. during the test period. Limitations of the supply of hormone precluded testing larger doses, but the previous study (Engel *et al.*, 1952) showed that even large doses of more potent growth hormone than used in the present work did not cause diabetes in the rat. 4. Cortisone plus growth hormone at 3 dose levels. The 2.2 mg. dose was tested approximately one year after the other doses when a small additional supply of this lot of hormone became available.

The blood glucose values in the various groups prior to the 5-day test period were essentially identical in all groups and did not differ significantly from those of day 4–5 in the tube-feeding control groups. These data are not included in Figure 3 or Table 2, but are recorded in Figure 1.

Figure 3 illustrates the results. None of the 3 control groups (no treatment, cortisone alone or growth hormone alone) exhibited significant glycosuria or hyperglycemia and none differed in any respect from the others. With combined growth hormone and cortisone treatment significant glycosuria and hyperglycemia developed with all doses of growth hormone tested when compared to any one of the 3 control groups. The glycosuria was increased as the dose of growth hormone was increased but there was no direct proportionality between the logarithm of the dose and the response. Thus, there were small but significant increments in glycosuria from the controls to the 0.55 and 1.1 mg. dose of growth hormone

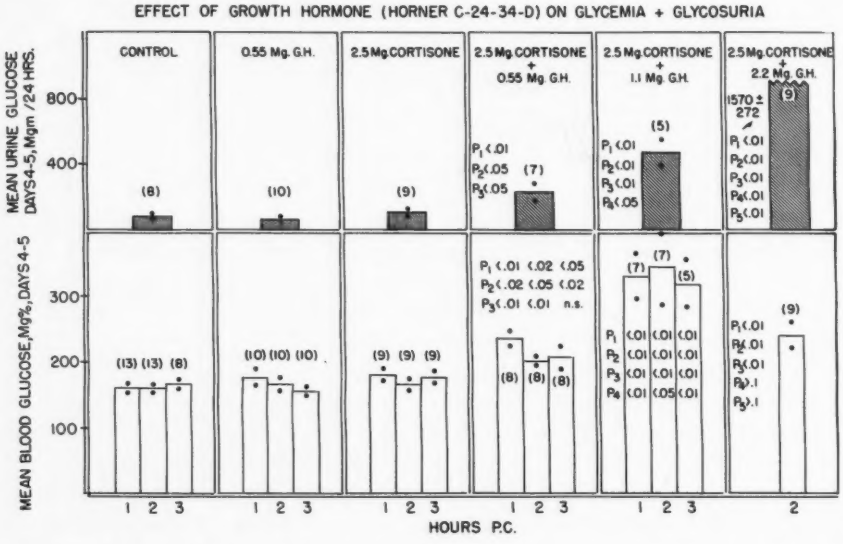

FIG. 3. Mean pooled blood glucose and urine sugar levels on the 4th and 5th days of treatment. See text for a description of the treatment groups. The P values refer to the probability of significance of the respective columns, compared to the previous columns, reading from left to right.

plus cortisone and then a three fold increase to 1570 mg. when the dose of growth hormone was doubled.

The blood glucose responses were equivalent at 1, 2 and 3 hours in each case and all but one of the blood sugar values for the growth hormone treated cortisone-primed rats were significantly higher than their corresponding controls. However, as with glycosuria, there was no consistent correlation between the dose of growth hormone and hyperglycemic response. The 1.1 mg. dose gave a response which was significantly greater

TABLE 2

Treatment	G.H. dose mg./100 gm.	No. of rats	Mean blood sugar ± SE 2 hours after feeding on days 4 and 5					
			mg. %	P†	Log mg. %	P†	% diabetes	P†
None	—	13	159± 6.4		2.20±0.018		8	
Cortisone Control	—	26	167± 3.9		2.22±0.010		8	
PR-1 Control	1.0	8	164± 6.8	N.S.	2.21±0.021	N.S.	0	N.S.
1. PR-1 Saline pH 3.5. HAC.	2.0	9	374±100.5	N.S.	2.46±0.103	<0.01	70[1]	<0.01
2. PR-1 Saline pH 3.5, Boiled	2.0	10	285± 55.0	N.S.	2.39±0.078	<0.01	50	<0.01
3. PR-1 0.2N HCl, Boiled	2.0	9	200± 33.0	N.S.	2.27±0.051	N.S.	33	N.S.
4. PR-1, NaOH, HCl, pH 3.5	1.0	10	237± 38.8	N.S.	2.34±0.055	<0.01	40	<0.05
4. PR-1 NaOH, HCl, pH 3.5	2.0	8	216± 11.9	<0.01	2.33±0.035	<0.01	70[2]	<0.01
5. PR-1 NaOH, HCl, pH 3.5, Boiled	2.0	8	198± 16.3	N.S.	2.29±0.034	<0.02	62	<0.01
6. PR-1 NaOH, HCl, pH 9.5	1.0	8	185± 18.9	N.S.	2.26±0.037	N.S.	25	N.S.
6. PR-1 NaOH, HCl, pH 9.5	2.0	11	214± 26.3	N.S.	2.30±0.046	<0.02	36	N.S.
7. PR-1 0.1N NaOH overnight	1.0	11	169± 9.8	N.S.	2.22±0.023	N.S.	27	N.S.
7. PR-1 0.1N NaOH overnight	2.0	7	164± 10.4	N.S.	2.21±0.027	N.S.	14	N.S.
Cortisone Control	—	9	164± 5.6		2.21±0.011		7	
C-24-34-D, pH 9.5 Control	0.55	10	166± 12.6	N.S.	2.21±0.031	N.S.	20	N.S.
C-24-34-D, pH 9.5	0.55	8	200± 8.4	<0.01	2.30±0.018	<0.01	50	<0.05
C-24-34-D, pH 9.5	1.1	7	353± 57.4	<0.01	2.50±0.063	<0.01	86	<0.01
C-24-34-D, pH 9.5	2.2	9	240± 19.7	<0.01	2.37±0.036	<0.01	78	<0.01
C-35-39-J, pH 9.5	0.4	3	154± 8.5	N.S.	2.19±0.023	N.S.	0	N.S.
C-35-39-J, pH 9.5	0.8	6	180± 11.3	N.S.	2.25±0.027	N.S.	33	N.S.
C-35-39-J, pH 9.5	1.6	7	276± 37.5	<0.01	2.42±0.065	<0.01	57	<0.01

[1] 10 rats—1 died with 4+ glycosuria.
[2] 10 rats—2 died with 4+ glycosuria.
† Significance compared to Cortisone Control.

than the 0.55 mg. dose, but the blood sugar level after the 2.2 mg. dose was not significantly different from either of the two smaller doses. The dissociation between hyperglycemia and glycosuria here is noteworthy. It has been noted before (Engel *et al.*, 1952) and suggests that the blood sugar elevation after the largest dose of hormone might persist during a greater portion of the day.

The blood sugar data were also calculated as the mean change in blood sugar levels between the 2 days prior to treatment and the 4th and 5th days of treatment in each group. Each animal served as its own control for this calculation and the mean difference for each group was calculated. No greater precision was achieved by this maneuver than by expressing the data as in Figure 3 and Table 2 and hence these data will not be recorded in detail here.

From this experiment it was concluded that hyperglycemia was the most sensitive indicator of diabetes since it was most apparent with the smallest dose of growth hormone and could be measured with greater precision than the urine sugar. There appeared to be no choice between the hour at which the blood sugar was examined, although other experiments, not recorded here, suggested that the 1 hour *post cibum* blood sugar level might be a little more variable than the 2 and 3 hour levels in the growth hormone treated animals. Furthermore, it was thought that artefacts due to epinephrine secretion or struggling during tube-feeding might be more apparent at 1 hour than later. For these reasons and for convenience in performing the experiment the 2 hour blood sugar was chosen as the criterion for diabetogenic activity in subsequent experiments.

It should be noted here that the above conclusions were reached before the 2.2 mg. dose of C-24-34-D was tested and on the basis of these conclusions urine sugar excretion measurements were not made in subsequent studies. The greater variability of the *post cibum* blood sugar of treated rats recorded in the following experiments and the rather marked accentuation of glycosuria by the 2.2 mg. of C-24-34-D without a corresponding change in 2 hour p.c. hyperglycemia indicate that the procedure adopted below may not have been the best choice. Limitations of growth hormone supply precluded further exploration of these points with the same lot of hormone and the available amounts of other lots were too small to permit repetition of the entire experiment.

Examples of the application of the procedure to samples of growth hormone. The procedure as described above, measuring the blood sugar levels 2 hours after feeding on days 4 and 5 of treatment and pooling the results, was then tested with two samples of growth hormone, PR1 and C-35-39-J. The results are recorded in Table 2 which also includes for comparison the data just described for growth hormone, C-24-34-D. For reasons noted below, the results were calculated and reported three ways for statistical analysis.

Sample PR1 had been prepared by the method of Raben and Westermeyer from pork pituitaries and, presumably, according to findings of these investigators, was expected to be low in, or free of, diabetogenic activity. In view of the findings of Reid (1953) that growth hormone prepared this way has less diabetogenic and growth activity when made up and injected at an acid pH than when injected at an alkaline pH, the hormone was prepared for injection at both pH 3.5 and pH 9.5. The effects of boiling and of alkali treatment were investigated as part of a comparative study of the effect of these various treatments on the growth, diabetogenic, adipokinetic and ketogenic activities in rats and mice and the hyperglycemic activity in man of this particular pituitary extract. These data are reported elsewhere (Engel, 1955). When one examines the blood sugar data from the studies with PR1 in Table 2, the results are disappointing since

in only one test was the blood glucose significantly higher than in the control. In this test the PR1 was dissolved in 0.1 N NaOH and immediately adjusted to pH 3.5 with HCl. On the other hand it is immediately apparent that the mean blood sugar levels in several groups were distinctly elevated, but were very variable and hence it must be assumed that at least some of the animals had become diabetic. Thus, for example, the blood sugar levels ranged between 137 and 925 mg.% in the 9 animals that received 2.0 mg. of PR1 in saline adjusted to pH 3.5 with acetic acid. It was obviously unreasonable to conclude on the basis of the "t" test that the hormone was not diabetogenic when, as in the group noted above, four animals showed blood sugar levels of 925, 840, 410 and 308 mg.% 2 hours after tube-feeding. Rather, it would appear that the data in this form are not susceptible to this type of statistical analysis since the distribution curve is skewed. Two alternative types of analysis were then tried. One was to perform a log transformation and analyze the resulting data by the "t" test. The other was to adopt an arbitrary criterion for diabetes and calculate the percentage of rats in each group that fell in this category, determining statistical significance by the chi-square test. The criterion of diabetes adopted was that the blood sugar should exceed the mean of the cortisone control plus two standard deviations, i.e., 167+40 or 207 mg.%. The application of these two manipulations brought out the actual occurrence and degree of diabetes in this study. Furthermore, with one exception, there was an excellent correlation between positive and negative results by the two methods of analysis, affording mutual support to their credibility. By these criteria it would appear that the diabetogenic activity of PR1 is not destroyed by boiling at pH 3.5, but is destroyed by boiling in 0.2 N HCl or by standing in 0.1 N alkali overnight. Furthermore, and in contrast to the experience of Reid with Raben-Westermeyer growth hormone, activity seemed to diminish when the extract was made up and injected at an alkaline pH. Further significance may be attached to these results by the finding, reported elsewhere (Engel, 1955) that the diabetogenic activity varied under the above circumstances in parallel with growth activity as tested by the tibial epiphysis test in hypophysectomized rats and with the ability of the extract to cause hyperglycemia following rapid intravenous infusion in man (Engel, 1955; Myers *et al.*, 1953). In contrast, the ketogenic and adipokinetic activities of PR1 resisted treatments with alkali which destroyed the growth and diabetogenic properties. Conversely, ketogenic and adipokinetic activities were lost on boiling at pH 3.5.

Data on C-24-34-D and C-35-39-J analyzed in the same fashions are recorded in the lower part of the table. These results were accumulated a year prior to those with PR1 and accordingly are compared to a different cortisone control. The mean +2 S.D. for this control was 198 mg.%. In contrast to the experience with PR1 the correspondence between the three types of statistical analyses was good. It is not known why the blood sugar

levels, in general, were less variable in this study than in the subsequent ones with a different lot of growth hormone.

DISCUSSION

The method as finally evolved is obviously in no wise an assay procedure in the strict sense of the term. On the other hand, as is apparent from the studies on PR1, it can be applied successfully to the problem of the simple detection of diabetogenic activity in pituitary extracts treated in various fashions and hence it has a practical application.

The failure to elicit any consistent correlation between the dose of hormone and the blood sugar response was disappointing, but is not at all surprising when one considers the end point chosen. The pathological state of diabetes induced by growth hormone is a variable one and cannot be compared to a physiological response such as growth of the tibial epiphysis. There are apparently variations in the susceptibility of the rats to the diabetogenic action of the hormone and in their ability to compensate, presumably by altering insulin secretion. Furthermore, the *post cibum* blood sugar is itself subject to a number of influences and may not correlate closely and directly with the degree of diabetes. This is apparent from the discrepancy between blood sugar values which represent the situation of the moment and glycosuria which is the reflection of the degree and time during which the blood sugar level exceeds the renal threshold during a 24 hour period. In this study the initial impression was obtained that the blood sugar response would be a more sensitive indicator of diabetes than the urine sugar and hence measurement of this latter parameter was not done in any of the later studies. On reviewing the entire study it appears that this impression may have been in error and that a suitable assay might still be developed on the basis of the glycosuric response.

Limitations in the supply of any given lot of growth hormone precluded a more extensive study of the many possible variables which might influence the test procedure. It is not to be assumed, therefore, that the conditions described for the test necessarily represent the optimal ones in terms of sensitivity, simplicity, or economy. Among the variables which might be explored by ourselves or other investigators when more pituitary growth hormone is available are the following: 1. Size and sex of rats. Relatively large rats were used in the original study on the suspicion that they might be more susceptible to a diabetogenic agent but this was never subjected to experimental verification. Smaller rats may be equally or perhaps even more sensitive. Their use would effect a considerable saving in hormone. Male rats were used because it has been our experience in other work in this laboratory that results with this sex are more consistent. It is not known whether there is a sex difference in sensitivity to the diabetogenic activity of growth hormone in this procedure. 2. Duration of

hormone treatment. The 5-day treatment period was adopted empirically and no attempt was made to see whether an earlier effect might be demonstrable. No exploration was made of the duration or maximal time of response with those doses of hormone just capable of bringing out diabetes. 3. Mode of administration of growth hormone. In the present study the hormone was given as a single subcutaneous injection in the morning. Would multiple injections or the suspension of the hormone in some menstruum allowing slow release be more effective? In view of the reported effect of growth hormone in stimulating release of the hyperglycemic factor of the pancreas and hence causing liver glycogenolysis (Bornstein, Reid and Young, 1951; Foa, Magid, Glassman and Weinstein, 1953; Werk, Kibler, Myers and Engel, 1955), is the blood sugar elevated one or two hours after injection for this reason and not because of diabetes? Since the hyperglycemic response to growth hormone mediated in this fashion is very transient we do not believe this factor to be significant, but believe nevertheless that it needs to be investigated further. 4. Diet. The high carbohydrate diet was chosen arbitrarily on the basis of the previous successful induction of diabetes in rats receiving this diet. Proportions of carbohydrate, protein or fat different from those used here might be more effective. A greater caloric intake might increase sensitivity since it is known that glycosuria may follow overfeeding alone (Ingle, 1946; Engel, Viau, Coggins and Lynn, 1952). 5. The number of times a rat may be used for test purposes. This point was not explored extensively but a few repeat tests on the same rat yielded reproducible results. In general, tests were not repeated sooner than one week after completion of the previous one. 6. Specificity. On the basis of the present study no claim can be made that the diabetogenic activity of the pituitary extracts tested resides solely in the growth hormone. Since ACTH itself can cause diabetes in the tube-fed rat and we have found that triiodothyronine will do likewise (Owen, Bogdonoff and Engel, 1955), this procedure may not distinguish between growth hormone, ACTH and TSH. ACTH is ineffectual unless given by repeated injections and hence it is unlikely that ACTH contamination presents a serious problem in our test procedure. The diabetogenic dose of triiodothyronine is very large and, paradoxically, when given with growth hormone the thyroid diabetes is decreased (Owen, Bogdonoff and Engel, 1955). It is unlikely, therefore, that a false positive response to TSH will result. Further investigation is necessary to determine whether any non-specific protein effects may result in animals receiving impure extracts. The ready destruction of the diabetogenic and growth activity of PR1 by chemical treatment suggests that this is not the case (Engel, 1955).

It is hoped that future work in this and other laboratories will clarify the many problems raised by this study. In the meantime, the technique

described is proving of sufficient reliability and reproducibility in this laboratory to permit its application to the problem of the purification of growth hormone and the study of its mode of action.

SUMMARY

1. The conditions have been established for the reproducible production of diabetes in the rat by the administration of anterior pituitary growth hormone. Rats adapted to high carbohydrate tube-feeding and primed with 2.5 mg. of cortisone acetate have been found suitable for this purpose.

2. The blood glucose level two hours after tube-feeding on the 4th and 5th days of combined hormone treatment appeared to be the most sensitive indication of the diabetic response. Once hyperglycemia was elicited there was a poor correlation between the degree of hyperglycemia and the dose of growth hormone administered.

3. A larger dose of growth hormone was necessary to induce glycosuria than hyperglycemia, but increases in the growth hormone dosage elicited more sharp increments in glycosuria than in hyperglycemia. Further investigation is indicated to determine whether the glycosuric response might be adapted for an assay procedure.

4. The technique has been applied with success to the study of the effects of chemical and physical treatments of a growth hormone preparation on its diabetogenic activity.

5. The limitations of the method are discussed and suggestions made for improving its sensitivity and precision.

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ADDENDUM

Since this study was completed and in press, it has been discovered that the high blood sugar levels recorded for groups 1 and 2, Table 2 are artefactual. Through an error, an inordinate amount of glacial acetic acid was added to the growth hormone PR-1 suspended in saline (0.7 ml. glacial acetic acid for each 50 mg. of PR-1 in 4.0 ml. saline). In the studies on growth and ketosis in the rat, adipokinesis in the mouse and hyperglycemia in man (Engel, 1955, Kibler, *et al.*, 1955) and subsequent studies on diabetes (Owen, Bogdonoff and Engel, 1955), 0.10–0.15 ml. of acetic acid was used for each 50 mg. of PR-1 suspended in 4.0–7.0 ml. of saline. In the growth assays and the hyperglycemia studies in man, the final concentration of acetic acid was much lower since the solutions were diluted considerably before injection. Preliminary studies indicate that the admixture of glacial acetic acid with an inert protein (human serum albumin) in the same proportions as used in groups 1 and 2 will cause a significant rise in blood sugar when injected into the cortisone-primed force-fed rat, but the final blood sugar values are significantly lower than in the case of the growth hormone preparation. For the purposes of the present study on alkali inactivation of PR-1, group 4 should be taken as the control.

HORMONAL AND NUTRITIONAL INFLUENCES ON THE BIOCHEMICAL COMPOSITION OF THE RAT TESTIS[1]

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STUDIES on the rat testis have included weight changes, spermatogenic activity and histochemical investigations. However, little attention has been directed toward the biochemical estimation of testis glycogen, lipid and nitrogen. The rat testis consists of approximately 85% water and 15% solids. The testis solids of adult rats are 65–70% protein (Addis, Poo and Lew, 1936; Aschkenasy and Dray, 1953; Leathem and Glasser, 1953) and approximately 30% lipid (Leathem and Wolf, 1955). Fresh testis tissue has yielded detectable glycogen (Leonard, 1952; Wolf, 1954). Testis protein is decreased by hypophysectomy and estrogen administration (Leathem and Glasser, 1953; Leathem, 1954; Greenberg and Leathem, 1954). However, fasting or feeding a protein free diet for 30 days does not deplete the adult rat testis of protein (Addis, Poo and Lew, 1936; Leathem, 1954). Nevertheless, maturation of the reproductive system is influenced by nutrition. In view of the paucity of information on the major components of the immature rat testis, the current study was undertaken. Various dietary proteins were fed to normal and to cortisone maintained adrenalectomized rats as factors possibly influencing testis composition.

MATERIALS AND METHODS

Immature male rats of the Long-Evans strain were adrenalectomized when 60–65 gm. Rats were fed Purina fox chow and a 20% protein diet (Table 1) in which casein, peanut flour and wheat gluten were the protein sources. In one experiment, a 5% casein diet was fed and was kept isocaloric with the other diets by replacing the protein with an equivalent amount of carbohydrate. Each kilogram of diet was supplemented with vitamins and 2 gm. of choline chloride (Table 1). Cortisone acetate (Cortone, Merck) in aqueous suspension was administered to all adrenalectomized rats during the experimental period of 20 days. Daily subcutaneous dosages of 0.15 mg. were used.

Rats were killed with an overdose of Nembutal injected intraperitoneally. Testes were weighed, then dried to constant weight at 95° C. Nitrogen and lipid content of the dry testes were determined by the methods of Pregl and Roth (1935) and Hsaio (1948) respectively. Glycogen concentration was determined on fresh tissue by the anthrone method (Seifter, *et al.*, 1950).

RESULTS

Testis weight gain was, in general, indicative of the nutritive value of the protein fed or the level of protein in the diet. The greatest gain in testis

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TABLE 1. COMPOSITION OF 20% PROTEIN DIET

	Per cent
Protein	20.0
Dextrose	20.0
Dextrin	25.7
Wesson Salt Mixture*	4.0
Cellu Flour	2.0
Crisco	25.0
Corn Oil	2.0
Choline Chloride	0.2
Vitamin Supplements/100 gm. of Diet	
Alpha tocopherol	4.0 mg.
Vitamin A	900 U.S.P. units
Vitamin D	180 U.S.P. units
2-methyl-1, 4 naphthoquinone diacetate	1.0 mg.
Thiamin HCl	0.8 mg.
Riboflavin	1.6 mg.
Pyridoxine HCl	0.8 mg.
Niacin	4.0 mg.
Calcium pantothenate	4.4 mg.
Para aminobenzoic acid	4.0 mg.
Inositol	21.0 mg.

* Modification of Osborn-Mendel salt mixture reported in Science **75:** 339. 1932.

weight was observed in intact rats fed either 20% casein in a purified diet or fox chow. A 5% casein diet permitted a slight testis weight increase but body weight failed to change. Replacing 20% casein with either 20% wheat gluten or 20% peanut flour resulted in a gain in body and testis weight which was significantly less than observed with casein (Table 2). Cortisone-maintained adrenalectomized rats gained less body weight than did intact rats fed identical amounts of the same diet, with the exception of rats fed 5% casein. A similar effect of adrenalectomy and cortisone was observed on testis growth when casein was fed. However, the subnormal testis growth rates observed with wheat gluten or peanut flour were not further depressed in adrenalectomized rats. (Table 2)

Concentration of testis protein was related to the percentage protein in the diet and the protein fed. Protein comprised 72.9% of the dry testis solids in rats fed 20% casein for 20 days, and was essentially unchanged from the initial concentration of 72.7%. Reduction of dietary casein from 20% to 5% caused a 10.3% decrease in testis protein concentration. Substituting wheat gluten or peanut flour for casein at the 20% level reduced testis protein by 8.1% and 6.6% respectively after 20 days. Thus a protein having a nutritive value less than that of casein not only slowed testis growth but also failed to maintain protein concentration.

Adrenalectomized rats maintained on cortisone exhibited testis protein concentrations which were less than those observed for intact rats. Limiting food intake of normal control rats to that of cortisone treated adrenalectomized rats revealed the adrenal-cortisone effect as essentially due to reduced food intake (peanut flour excepted).

Total testis protein was influenced by testis size and by protein concentration. Again the casein fed rats exhibited the greatest amount of total

TABLE 2. HORMONAL AND NUTRITIONAL EFFECTS ON IMMATURE RAT TESTIS PROTEIN AND LIPID

Treatment	Diet	No. of rats	Final body wt., gm.	Testis wt., gm.	Water %	Protein			Lipid		
						%	Total, gm.	gm./100 gm. body wt.	%	Total, gm.	gm./100 gm. body wt.
Control[1]	20% Casein	8	129.2± 6.3	1.529± .118	85.0± .41	72.9± 1.2	.140± .003	.103± .012	30.4± .44	.074± .002	.049± .001
Adx[2]+E ac[3]		9	76.6± 1.7	.703± .119	86.0± .31	68.5± .61	.102± .007	.131± .003	30.4± .24	.043± .003	.046± .010
Control[4]		7	113.1± 4.1	1.211± .138	85.8± .33	70.5± .93	.133± .006	.120± .009	29.4± .77	.063± .007	.052± .001
Control[1]	5% Casein	7	60.3± 1.8	.763± .104	86.1± .17	62.4± .39	.076± .001	.121± .005	29.2± .20	.032± .001	.053± .003
Adx[2]+E ac[3]		8	58.4± 1.7	.688± .069	85.7± .14	60.3± .80	.067± .009	.103± .013	28.9± .34	.019± .002	.049± .001
Control[4]		8	60.1± 1.8	.529± .088	88.4± .26	61.5± .76	.045± .003	.075± .010	28.9± .19	.022± .001	.037± .001
Control[1]	20% Wheat Gluten	7	83.5± 2.8	.959± .136	88.3± .62	64.6± .32	.110± .007	.133± .002	34.0± .21	.059± .009	.071± .010
Adx[2]+E ac[3]		8	58.1± 3.2	.808± .087	87.1± .51	61.0± .83	.062± .003	.108± .002	31.8± .43	.031± .014	.053± .017
Control[4]		7	77.6± 2.6	.816± .099	87.4± .43	62.3± .56	.065± .012	.086± .018	32.2± .34	.035± .010	.046± .004
Control[1]	20% Peanut Flour	6	81.2± 1.7	1.051± .182	85.2± .20	66.1± .73	.118± .021	.146± .013	28.8± .31	.044± .001	.053± .001
Adx[2]+E ac[3]		9	59.1± 1.7	1.014± .113	84.1± .41	64.3± .81	.102± .001	.169± .002	31.3± .37	.064± .001	.107± .003
Control[4]		6	72.5± 2.5	.699± .093	86.2± .14	68.5± 1.4	.101± .005	.141± .010	31.3± .24	.045± .002	.068± .002
Initial Control	Fox Chow	8	62.1± 1.7	0.405± .046	85.4± .12	72.7± .25	.080± .005	.133± .022	30.1± .50	.033± .010	.053± .010
Final Control		6	109.0± 3.1	1.221± .126	86.4± .31	70.3± .88	.105± .020	.092± .011	30.3± .55	.054± .010	.050± .009

[1] Fed *ad libitum*.
[2] Adrenalectomized.
[3] Cortisone acetate, daily dosage 0.15 mg.
[4] Food intake limited to adrenalectomized rats.

testis protein; however, rats fed peanut flour had a greater amount of testis protein in relationship to body weight.

Concentration of testis lipid was not influenced by protein source, level of protein fed or adrenalectomy (Table 2). A tendency toward higher fat concentration was obtained when wheat gluten was the protein source. Total testis fat varied with testis size. Testis fat per 100 gm. of body weight was increased in normal rats fed wheat gluten and in the group of adrenalectomized rats fed peanut flour.

Testis glycogen (Table 3) was not influenced by adrenalectomy, protein source or by a restricted food intake; although rats fed a wheat gluten diet tended to have a higher glycogen concentration than those fed a 20% casein or peanut flour diet. A definite increase, however, in glycogen per gram of tissue was observed in intact rats when the level of dietary casein was reduced from a 20% level to 5%. The increase was less dramatic when cortisone-maintained adrenalectomized rats and their food restricted controls were fed the reduced level of protein.

DISCUSSION

Adequate nutrition is known to be of importance to the reproductive system (Ershoff, 1952). The results of these experiments indicate that testis weight gain and the biochemical composition of the immature rat

TABLE 3. HORMONAL AND NUTRITIONAL EFFECTS ON IMMATURE RAT TESTIS GLYCOGEN

Treatment	Diet	No. of rats	Final body wt. gm.	Testis wt. gm.	Glycogen		
					%	Total mg.	mg./100 gm. body wt.
Control*	20% Casein	7	128.6 ± 4.2	1.468 ± .183	0.11 ± .04	1.75 ± .23	1.35 ± .16
Adx + E ac†		9	77.0 ± 1.1	1.025 ± .334	0.13 ± .04	1.21 ± .08	1.59 ± .10
Control‡		8	113.0 ± 5.6	1.170 ± .234	0.15 ± .02	1.83 ± .19	1.59 ± .13
Control*	5% Casein	8	61.0 ± 1.8	0.684 ± .151	0.26 ± .08	1.76 ± .33	2.88 ± .26
Adx + E ac†		7	59.2 ± 3.1	0.553 ± .073	0.23 ± .07	1.26 ± .42	2.18 ± .11
Control‡		7	61.4 ± 3.3	0.591 ± .082	0.23 ± .03	1.35 ± .21	2.28 ± .48
Control*	20% Wheat Gluten	8	82.3 ± 2.5	1.017 ± .204	0.18 ± .04	1.66 ± .21	2.00 ± .22
Adx + E ac†		8	58.0 ± 3.8	0.537 ± .112	0.17 ± .03	1.05 ± .42	1.81 ± .34
Control‡		8	73.0 ± 2.1	0.871 ± .081	0.17 ± .01	1.49 ± .27	2.07 ± .34
Control*	20% Peanut Flour	5	80.8 ± 1.2	1.257 ± .178	0.11 ± .05	1.35 ± .30	1.61 ± .06
Adx + E ac†		8	60.7 ± 1.4	.923 ± .195	0.17 ± .04	1.59 ± .41	2.72 ± .33
Control‡		7	70.9 ± 2.2	0.780 ± .255	0.12 ± .02	1.04 ± .27	1.59 ± .19
Initial Control	Fox Chow	7	61.0 ± 2.3	.273 ± .032	0.19 ± .01	0.528 ± .16	0.877 ± .27
Final Control		8	115.1 ± 3.6	1.515 ± .255	0.15 ± .03	2.14 ± .45	1.86 ± .31

* *Ad libitum.*
† Daily dosage cortisone acetate 0.15 mg.
‡ Food intake limited to adx rats.

testis may be influenced not only by the percentage of protein in the diet but also by the nutritive value of the protein fed. Of the proteins studied, casein, peanut flour and wheat gluten would be listed in decreasing order of biological value (Allison, 1953). Our data on testis protein concentration indicates a similar order of nutritive value for these proteins.

These data are in contrast to previous reports on adult rats (Addis, *et al.*, 1936; Leathem, 1954) which indicated that testes failed to yield protein during either a fast or during a 30-day period of protein deprivation. It would appear that in immature rats either the protein stores are more

labile in that they markedly reflect changes in dietary protein or that in the growing rat, testis protein assimilation is dependent upon the nutrition offered to the rat.

Histochemical data have shown that gonadal lipid may be accumulated following either hypophysectomy or dietary retardation (Lynch and Scott, 1951). Lipid analyses of the immature testis, however, showed no significant difference when the various proteins were fed. This apparent discrepancy between the qualitative and quantitative data may well be the result of the shorter experimental period or, that while the nutrition offered the animals was sufficient to alter protein concentration, it was not of such a poor quality as to favor lipid accumulation. Leathem (1954) reported the accumulation of testis lipid when adult rats were protein-depleted for 30 days, hypophysectomized, and then continued on protein depletion or refed 18% casein for 20 additional days.

Although histological study has revealed mucopolysaccharides in rat testes, glycogen has not been found (Leblond, 1950). Our data show that glycogen is present in the rat testis but its concentration could be altered only by a sharp reduction in dietary protein.

SUMMARY

The immature rat testis contains approximately 85% water, 10.5% protein, 4.5% lipid and detectable glycogen. Testis composition was maintained for 20 days in rats fed 20% casein. Proteins of lower nutritive value (wheat gluten, peanut flour) decreased testis protein concentration and weight gain, increased percent water but in general did not influence lipid or glycogen. A 5% casein diet reduced testis protein and increased glycogen concentration. Cortisone-maintained adrenalectomized rats exhibited a testis protein loss due largely to reduced food intake but testis glycogen was unaffected.

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THE EFFECT OF TESTOSTERONE PROPIONATE ON MUSCLE GLYCOGEN IN ALLOXAN DIABETIC RATS[1]

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GROWTH hormone stimulates glycogen deposition in skeletal muscles of normal and hypophysectomized rats, but not in alloxan diabetic rats (Illingworth and Russell, 1951). Since testosterone acts similarly to growth hormone in increasing glycogen stores in skeletal muscles (Leonard, 1952 a,b), an investigation was made to determine if a normal insulin supply is required for glycogen deposition by testosterone. In view of the differences in behavior of different skeletal muscles to glycopexic hormones (Leonard, 1952b, 1953), three representative muscles were studied, namely the rectus femoris, diaphragm and levator ani.

METHODS

Adult normal and castrated male rats of the Long-Evans, strain weighing between 200–300 gm., were used. Diabetes was produced by the administration of alloxan monohydrate (175 mg./kg. body weight) intraperitoneally, after a 72 hour fast. In the castrated rats, alloxan was administered 23 days after castration. Four days later, blood samples from the tail vein were drawn and blood sugar levels determined by the Somogyi (1945) modification of the Nelson method (1944). Animals with non-fasting blood sugar levels of less than 250 mg.% were not considered diabetic and were discarded.

Testosterone propionate[2] in oil was injected subcutaneously in 1 mg. doses for 3 days beginning on the fourth day after alloxan treatment. All animals were starved 24 hours prior to autopsy. Under Nembutal anesthesia the muscles were removed in the following order: rectus femoris, levator ani and diaphragm. Glycogen determinations were made by the anthrone method of Seifter *et al.* (1950). In addition, observations were made on the relative weight changes in the levator ani muscle.

RESULTS

Observations were first made on changes in the muscle glycogen levels of intact diabetic rats, 7 days after the injection of alloxan. The results in Table 1 show no change in the glycogen concentrations in the rectus femoris and diaphragm but a significant decrease in the levator ani.

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[2] The testosterone propionate used in this study was furnished through the generosity of the Schering Corporation, Bloomfield, New Jersey.

Following castration, the glycogen levels in the three muscles were less than that in the normal controls, although in the diaphragm, the decrease was not statistically significant. When the castrated rats were made diabetic there was a tendency for the glycogen levels to increase in the rectus femoris and diaphragm muscles; however, in the levator ani the glycogen concentration was slightly lower.

The administration of testosterone propionate to castrated rats markedly increased the glycogen levels above that observed in the castrated controls. The increase was 38%, 69% and 73% for the rectus femoris, diaphragm and levator ani respectively. When the castrated rats were made diabetic and injected with testosterone, the glycogen stores in the rectus femoris and

TABLE 1. THE EFFECT OF TESTOSTERONE ON MUSCLE GLYCOGEN IN DIABETIC RATS

Experiment	No. of rats	Glycogen (mg. per 100 gm. muscle)		
		Rectus femoris	Diaphragm	Levator ani
Normals	7	466 ± 15*	333 ± 8	504 ± 18
Intact Diab.	9	460 ± 26	312 ± 14	303 ± 20
Castr.	7	394 ± 15	292 ± 18	418 ± 24
Castr. Diab.	9	460 ± 28	368 ± 17	344 ± 31
Castr. + Testos.	7	543 ± 14	494 ± 23	721 ± 28
Castr. Diab. + Testos.	11	494 ± 32	372 ± 33	865 ± 36

* Standard error of the mean in this and subsequent table.

diaphragm did not increase above that found in the castrate-diabetic control. In contrast to this, the glycogen concentration in the levator ani increased 151% above the castrate-diabetic control. This concentration was the highest obtained in any of the experiments.

The increase in weight of the levator ani muscle resulting from treatment with androgens has been suggested as a measure of the anabolic property of these steroids (Eisenberg and Gordon, 1950). Therefore, the weight changes of this muscle in these experiments was carefully noted, particularly to observe what effect the diabetic state would have on the growth response. Table 2 shows that alloxan diabetes in normal rats induced a marked drop in the weight of the levator ani. Castration alone produced an even greater loss of weight which was not lowered further by the imposition of diabetes on the castrated rats. The injection of testosterone in the castrated and castrated-diabetic rats increased the weight of this muscle to the same extent in both groups.

DISCUSSION

These experiments indicate that testosterone did not elevate the glycogen levels in the rectus femoris and diaphragm muscles of diabetic rats, under conditions where there is a relative insufficiency of insulin. Similarly, growth hormone failed to stimulate the deposition of glycogen in the

gastrocnemius muscle and diaphragm of the alloxan diabetic rat (Illingworth and Russell, 1951). However, testosterone markedly increased the glycogen concentration in the levator ani muscle of the diabetic rats and it is assumed that a normal insulin supply is not necessary in this instance. There is no explanation why testosterone failed to increase the glycogen content of certain muscles under a condition of relative insulin insufficiency.

Alloxan diabetes in the intact rat had no effect on the glycogen levels in the rectus femoris and diaphragm muscles but caused a fall in the levator ani. Castration decreased the glycogen in all muscles although the amount was insignificant in the diaphragm. The imposition of diabetes on the castrated animals produced a rise in muscle glycogen except in the levator ani. Previous reports indicate that muscle glycogen decreased following alloxan

TABLE 2. GROWTH RESPONSE OF THE LEVATOR ANI TO TESTOSTERONE IN RELATION TO DIABETES

Experiment	No. of rats	Weight of levator ani (mg. per 100 gm. body weight)
Normals	7	62 ± 2
Intact Diab.	9	33 ± 2
Castr.	7	24 ± 2
Castr. Diab.	9	24 ± 1
Castr. + Testos.	7	37 ± 3
Castr. Diab. + Testos.	11	39 ± 2

diabetes (Lackey *et al.*, 1944; Bornstein and Trewhella, 1950; Cullimore *et al.*, 1953) but Illingworth and Russell (1951) found the glycogen levels within the normal range.

A consideration of the changes in the glycogen levels in different muscles, following the induction of diabetes may involve two possible factors; (1) alterations in adrenal cortical activity (2) the effect of increased blood sugar levels. Alloxan is toxic and produces a stress as indicated by an enlargement of the adrenal cortex (Morita and Orten, 1950) and presumably stimulates an increased secretion of the cortical steroids. The cortical steroid, cortisone, exhibits a glycopexic effect in the rectus femoris and diaphragm muscles which is particularly noticeable when the initial glycogen level is lowered by other means. However, cortisone is not glycopexic for certain muscles (the cremaster), peculiarly sensitive to the male hormone (Leonard, 1953). The levator ani is similar to the cremaster in that it also fails to respond to cortisone as shown by the following experiment. The average glycogen concentration in the levator ani muscle of 6 castrate rats treated with 2 mg. of cortisone daily for 3 days was 388 ± 12 mg.% and that of 6 castrated controls was 363 ± 10 mg.% Therefore it is possible that the increased secretion of the cortical hormones as a result of alloxan treatment elevated the glycogen in only the rectus femoris and diaphragm muscles in the castrated rats. A similar mechanism can explain the maintenance of the muscle glycogen levels in intact rats made diabetic.

The loss of glycogen from the levator ani muscle of the intact rats given alloxan is apparently due mainly to the decreased production of male hormone as shown by the loss of weight of this muscle (Table 2). The growth of muscles of the perineal complex, which includes the levator ani, is dependent on testosterone (Wainman and Shipounoff, 1941). Since alloxan is known to injure the pituitary gland structurally (Ruben and Yardumian, 1946) this could result indirectly in a decreased production of the male hormone in the intact diabetic rats.

It is questionable whether the elevated blood sugars of the castrated-diabetic rats would account for the higher muscle glycogen levels in these animals as compared to the castrated rats. The "mass action effect" of the excess sugar, as suggested by Morita and Orten (1950), might explain the higher glycogen levels. The administration of glucose to alloxan diabetic rats was reported to increase the deposition of muscle glycogen (Barker and Sweet, 1937; Weber, 1946). On the contrary, Illingworth and Russell (1951) showed that fed glucose did not increase the muscle glycogen stores in diabetic rats. Cullimore *et al.* (1953) could not correlate the blood sugar levels with muscle glycogen levels in rats with alloxan diabetes of four weeks duration. If it is a matter of simple equilibrium between high blood sugars and elevated muscle glycogen, then all of the muscles of the castrated rats might be expected to show a rise in glycogen on making them diabetic. This was not true for all muscles. The levator ani failed to respond.

Finally, the data show that a normal insulin supply is not necessary for the growth of the levator ani when stimulated by testosterone. The relative increase in weight of this muscle was the same in castrated and castrated-diabetic rats.

SUMMARY

Studies were made on the changes in glycogen concentration in the rectus femoris, diaphragm and levator ani muscles of castrated rats, castrated rats treated with testosterone, intact diabetic, castrated-diabetic, and castrated-diabetic rats treated with testosterone.

No change occurred in the glycogen concentration in the rectus femoris and diaphragm muscles of intact male rats made diabetic with alloxan, but the glycogen in the levator ani decreased. Castration decreased the glycogen concentration in these muscles but the imposition of diabetes on the castrated rats increased the glycogen in the rectus femoris and diaphragm muscles but not in the levator ani.

Testosterone, which increased the glycogen in all three muscles in castrated rats, failed to increase the glycogen in the rectus femoris and diaphragm of the castrated-diabetic rats. In the levator ani, however, testosterone markedly increased the glycogen level.

The growth response of the levator ani to testosterone was not influenced by the diabetic condition.

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THE PROTEIN ANABOLIC AND OTHER EFFECTS OF TESTOSTERONE PROPIONATE IN MICE: EFFECTS OF NUTRITION AND INTERRELATIONSHIP OF BIOLOGIC ACTIVITIES OF THE HORMONE[1]

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INTRODUCTION

THE conventional assays for the biologic activities of testosterone propionate concern themselves with measurement of its effects on secondary sex characteristics such as the growth of the capon or chicken comb, the weight of the seminal vesicles, prostate, and preputial gland in the castrate rat, (Dorfman, 1950).

With increasing awareness of the clinical usefulness of the so-called "independent" actions of such sex hormones, methods for the quantitative evaluation of some of these effects have been devised (Selye, 1949). Such studies include those of Kochakian (1950) on body weight and nitrogen excretion of rats, those of Papanicolaou and Falk (1938) on the growth of the masseter muscle in the guinea pig, those of Eisenberg and Gordon (1950) on the levator ani muscle of the rat, and previous work from this laboratory (Homburger *et al.*, 1950) on the behavior of the clitoris and the normal and hydronephrotic kidney of white Swiss mice, and on the stimulation of renal β-glycuronidase activity in mice under standard conditions (Fishman, 1951 and 1953).

The question of the interdependence of these various effects of a single synthetic compound is of great theoretical interest. A separation of the sexual from the other effects is desirable from a clinical point of view.

Previous work has shown that a number of synthetic derivatives of testosterone have biologic effects which differ from each other. Thus it has been claimed, for instance, that methylandrostenediol is relatively more myotropic than virilizing (Eisenberg and Gordon, 1950, Homburger *et al.*, 1953).

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This paper presents an attempt to explore the possibility of using the white Swiss mouse for studies on growth-promoting properties of testosterone propionate, and to evaluate the effects of dietary regimens on some of the actions of testosterone propionate in these animals. It will be seen that some of the effects of the hormone are dependent on adequate nutrition and may be masked by deficient diets, whereas others are not. Thus evidence is provided in favor of the concept that some of the actions of testosterone propionate can occur entirely independently from others.

METHODS

Immature female mice were used in these experiments. They were obtained from two different sources: from Rockland Farms and from our own breeding colony, which is heterogeneous, being derived from various lines. The use of intact females three weeks old gave the most pronounced effects.

The following variables were studied in separate experiments:

(1) A normal diet with restricted intake.*
(2)A normal diet with unrestricted intake.
(3) A high-protein diet.†

The experiments were continued for one month, during which time the mice were injected subcutaneously every other day with 2 mg. of testosterone propionate in peanut oil and then weighed. An equal number of control animals received peanut oil injections only. In each experiment the mice were divided into groups of ten to twelve, with no more than six to a cage. The food was presented according to the specifications of the particular experiment.

In the mice whose diet was restricted, the amount fed was made equal to the lowest food intake of any group in the particular experiment (usually the controls), and the entire group was offered this amount on the following day. After several days this amount became fairly constant. Water was given ad libitum, and the animals were housed under routine conditions.

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* This diet was composed as follows:

Pulverized Fox Checkers	50 parts
Vitamin B-free synthetic ration (General Biochemicals, Inc.)	50 parts
Cod liver oil	1 part
Vitamin B:	
Thiamin	5 mg./mouse/day
Riboflavin	40 mg./mouse/day
Pyridoxin	10 mg./mouse/day

To this mixture was added enough water to make a paste, which was placed in non-scatter food cups.

† This diet, devised by Kochakian and Van Den Mark (1952), was composed as follows:

	gm.
Casein	46.4
Sugar	31.5
Vegetable fat (hydrogenated)	7.4
Salts	3.7
Ruffex	1.8
Yeast	9.2

For ten mice, 10 drops of cod liver oil and 1 drop of wheat germ oil were added to each two-day batch of food.

On the twenty-eighth day of the experiment, after having been deprived of food for twenty-four hours, the animals were killed by cervical dislocation and autopsied. The weights of the following tissues were measured on Smith roller torsion balances: clitoris, adrenal gland, carcass, and both kidneys.

In addition to this autopsy data, the following determinations were made:

(1) The kidneys and carcasses were weighed after being dried in an oven to constant weight at 105 to 110°, one kidney from each mouse being used.
(2) β-glucuronidase determinations were made on aqueous homogenates of kidney according to methods described elsewhere.
(3) Histologic studies were made of the remaining kidneys, after the samples for the enzyme determination had been taken.

RESULTS

The results of the experiments were as follows:

Increase in Body Weight. As shown in Figure 1, a statistically significant difference in the rate of growth was observed in the Rockland Farms mice, mice, which were well fed compared with those from the same source on a restricted diet. However, there appears to be a difference in the sensitivity of mice to testosterone in this respect, depending on their source, since in contrast to the Rockland Farms mice, those from our breeding colony did not exhibit a noticeable growth response. The reason for this has not been determined. Mice from our colony fed a high-protein diet showed no acceleration of growth rate.

The other effects of testosterone propionate, summarized in Figure 2 were as follows:

Renotropic Effect. This effect was noted in every case in which an animal was treated with testosterone. It was most marked in the animals fed an unrestricted diet. The further increase of protein in the diet did not increase the renal response. This was true for increases in kidney weight as well as for renal β-glucuronidase activity.

Clitoritropic Effect. This was observed in all animals injected with testosterone, regardless of diet.

Adrenal Inhibitory Effect. This was pronounced in all animals injected with testosterone and did not depend on the dietary intake.

Water Content of Kidney and Carcass. The testosterone-treated animals on the normal diet, whether unrestricted or restricted, had a higher water content in the carcass than did the controls, all P values being less than .05. There was also a greater amount of water in the kidneys of the entire group of well-fed animals, both control and test, compared with those on the restricted diet, with P values below .01. In Figure 2 this is not evident, all controls having been set arbitrarily at 100%. The high-protein diet had no effect on the water content of the carcass and kidney.

DISCUSSION

These studies show that in immature white Swiss mice the effect of testosterone propionate on the growth rate as expressed in terms of body weight curves can be dependent on the food intake maintained during the

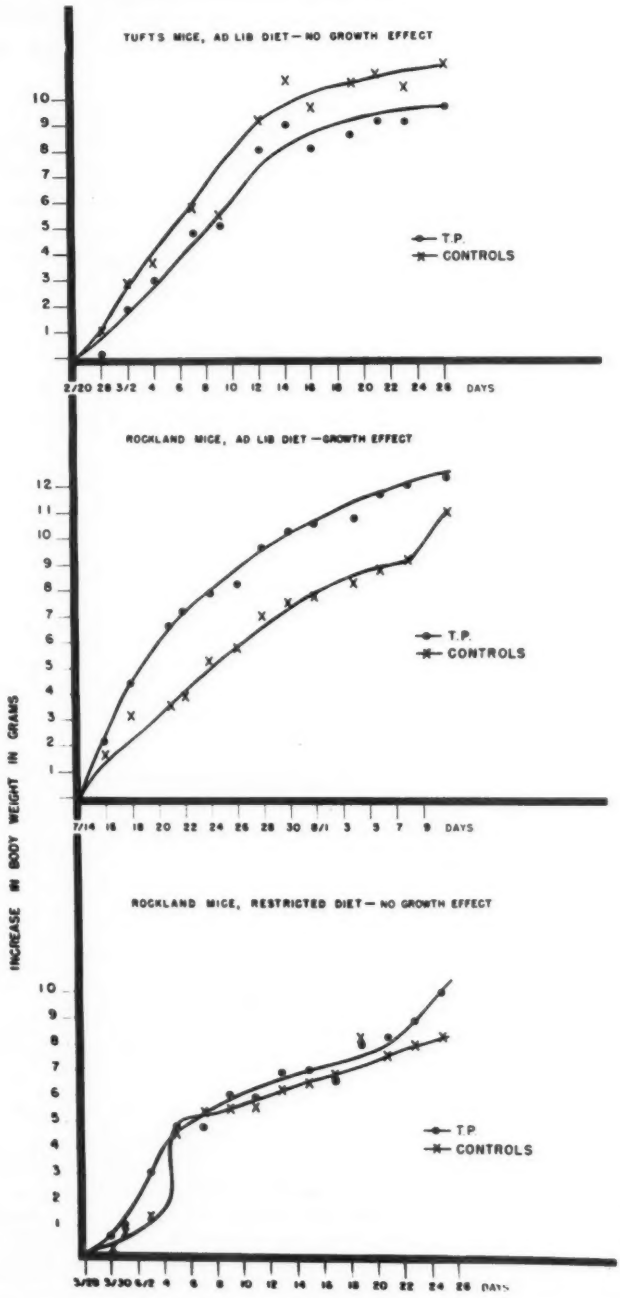

FIG. 1. Showing the effects of dietary restriction and type of mice upon the weight increasing action of testosterone propionate.

experiment, and possibly on other factors having to do with the genetic makeup of the animals or their environment previous to the start of the experiment.

The per cent water determinations on kidney and carcass have been included in an attempt to present another piece of evidence, although indirect, for the protein anabolic action of testosterone propionate in addition to the observation of an increased growth rate. Kochakian (1950) reports an increase in water content of tissues in testosterone-treated animals and attributes it to the increase in tissue protein at the expense of fat. He finds no differences when the weights are determined on a fat-free basis.

The positive values obtained in measuring the water in the organs show some correlation with the appearance of an increased growth rate. This may be a measure not only of the anabolic action of testosterone but also of the general nutrition of the animal.

Testosterone had no effect on body growth while the animals were receiving a restricted diet. Thus this effect did not parallel the other effects of the steroid. These latter effects, such as renotropism, expressed by kidney weight and increased activity of renal β-glucuronidase, virilization as expressed by the increase in size of the clitoris, and reduced weight of the adrenal gland, occurred even in those animals which failed to grow at a

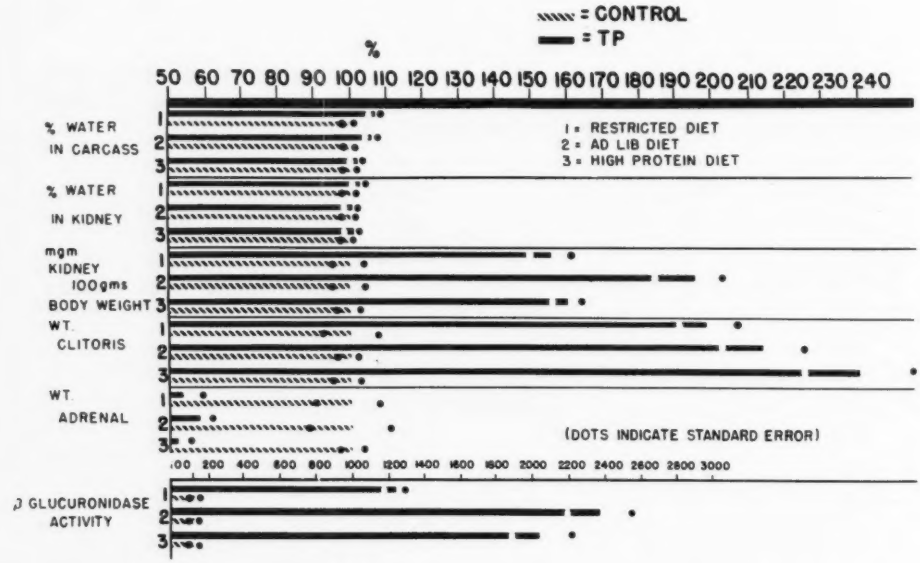
= CONTROL
= TP
%
50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240
% WATER IN CARCASS
% WATER IN KIDNEY
mgm KIDNEY 100gms BODY WEIGHT
WT. CLITORIS
WT. ADRENAL
1 = RESTRICTED DIET
2 = AD LIB DIET
3 = HIGH PROTEIN DIET
(DOTS INDICATE STANDARD ERROR)
200 400 600 800 1000 1200 1400 1600 1800 2000 2200 2400 2600 2800 3000
β GLUCURONIDASE ACTIVITY

FIG. 2. Comparison of various organ weights of mice on three experimental diets. Values have been converted to a percentage basis, the controls having been arbitrarily assigned a 100% value.

faster rate under the influence of testosterone. Hence, these last three biologic properties appear to be less dependent on the level of food intake than is the growth rate. These experiments demonstrate only that some of the effects of testosterone other than body growth stimulation may occur more readily and not necessarily simultaneously with increased body growth. The reverse, that growth stimulation could occur without any of the other effects, has not been demonstrated.

It is suggested by these observations that failure of testosterone propionate to accelerate growth rates in animals may be due to experimental conditions such as food intake, genetic background, and prior environment. The importance of controlling all these extraneous factors when evaluating the effects of any substance on growth cannot be over-emphasized.

SUMMARY

Various biologic properties of testosterone propionate were studied in female white Swiss mice under varying conditions.

The principal activities studied were effect on body weight increase and water content of the carcass, renotropism as measured by kidney size, renal β-glucuronidase activity, virilization as measured by the increase in weight of the clitoris, and adrenal depressor effect as measured by the reduction of adrenal gland weight. The variables introduced into the experiments were (1) the source of the animals (Rockland Farms versus our own colony); (2) the level of dietary intake (unrestricted quantities of a normal diet versus restricted amounts of the same diet); and (3) variations in the composition of food (normal protein content versus high-protein content).

Renotropism, virilization, and adrenal depressor effect occurred to almost the same degree under all experimental conditions, whether or not the animals were growing at an increased rate. Conversely, the growth stimulation of testosterone propionate in the mice on a normal diet depended on the source of the animals and did not occur either on a restricted intake nor on a high-protein diet.

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FURTHER OBSERVATIONS ON PITUITARY STRUCTURE AND FUNCTION IN RATS BEARING HYPOTHALAMIC LESIONS[1,2]

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IN AN earlier report (Bogdanove and Halmi, 1953) we presented histophysiologic observations on the pituitary and target glands of rats which had been treated with propylthiouracil (PTU) following the placement of hypothalamic lesions. We were able to confirm Greer's (1951, 1952) finding that certain hypothalamic lesions may prevent or impair PTU-induced thyroid hyperplasia. However, we never observed actual atrophy of this gland, and the question arose whether similar lesions would produce atrophy and/or decreased function of the thyroid in rats which did not receive any goitrogen. Further, since goitrogen treatment itself results in degranulation of the acidophils and thyrotrophs of the adenohypophysis, the effects of hypothalamic lesions *per se* on these cell types could not be determined in PTU-treated rats. These unsolved problems prompted the currently reported investigation of the effects of various hypothalamic lesions on rats which did not receive PTU.

In the goitrogen-treated series we had found, in accordance with several previous investigators (Harris, 1948), that testicular atrophy (resembling that seen after hypophysectomy) occurred in a number of the lesion-bearing rats. Since the pituitary gonadotrophs of such animals were either absent or showed marked regressive changes, it was logical to assume that the testicular involution was secondary to a failure of gonadotrophin secretion. In the present study, to examine this problem further, we have assayed the gonadotrophin content of pituitary tissue from normal rats and from lesion-bearing rats with and without gonadal atrophy, correlating the finding with morphological observations on the same glands.

Our earlier work indicated that hypothalamic lesions may impair the gonadotrophic and thyrotrophic mechanisms of the adenohypophysis more

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[2] A preliminary report of these studies has appeared in abstract form (Bogdanove, 1953).

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or less selectively. In the present study, we have sought to determine whether such lesions can interfere with the characteristic response of the hypophysial gonadotrophs to castration, and whether such an interference, if it does occur, necessarily is accompanied by changes in the responsiveness of the pituitary-thyroid axis to chemically induced hypothyroidism. To this end, lesion-bearing rats (with and without testicular atrophy) were castrated and subsequently subjected to a goitrogenic regimen.

The present report also includes the observation of adrenal atrophy in some of the animals with hypothalamic lesions.

MATERIAL AND METHODS

Bilateral electrolytic hypothalamic lesions were placed, by means of a Krieg-Johnson stereotaxic apparatus, in young adult male Sprague-Dawley rats (initial weights 200–340 gms.) under ether anesthesia. The animals were maintained on tap water and Rockland pellets *ad libitum*. Forty-five of the rats (group I) were sacrificed between 29 and 50 days after the operation, whereas 11 (group II) were maintained for 73 to 90 days. Seventeen rats with lesions (group III) were castrated and treated as described below.

At autopsy the pituitaries of 19 rats from group I (as well as those of 16 intact controls) were carefully divided in the mid-sagittal plane. One half of each gland was homogenized in 3 ml. of physiologic saline for assay of its gonadotrophin content, whereas the other was processed for histologic study. The saline homogenate of each half-gland was administered (in 6 subcutaneous injections, 12 hours apart) to one immature female rat (24–26 days old at the onset of the assay). Between injections the homogenates were stored at 4° C. Twelve hours after the last injection the recipients were sacrificed and their ovaries and uteri (minus contents) weighed on a torsion balance. Ten uninjected littermate females served as recipient controls.

Thyroid-serum radioiodide concentration ratios were determined in 8 rats of group I and all rats of group II by the procedure previously described (Halmi *et al.*, 1953).

The rats of group III, together with 5 intact controls, were castrated (under nembutal anesthesia) approximately one month after the hypothalamic intervention. The testes were weighed and examined histologically. Starting 33–34 days after orchidectomy, these 22 animals were injected daily for 20–21 days with 10 mg. of propylthiouracil (in aqueous suspension with gum acacia). They were sacrificed 24 hours after the last injection.

At autopsy, the whole or half pituitaries from every rat, and pancreatic tissue from some, were fixed without weighing in SUSA-picric acid (9:1). Testes, seminal vesicles, ventral prostates, thyroids and adrenals were weighed on a torsion balance and fixed in the same fluid. Brains were fixed in 10% neutral formalin for lesion localization studies, which do not form part of this report. All tissues were embedded in Tissuemat. Pituitary sections, cut at 4 μ and 6 μ in the horizontal plane, were taken from at least 3 levels of each gland and mounted together. The 4 μ sections were stained with Gomori's trichrome and with aldehyde fuchsin-trichrome (Halmi, 1952a) and those cut at 6 μ with periodic acid-Schiff (PAS)-hemalum as applied by Purves and Griesbach (1951). The other organs were sectioned at 6 μ and stained with Gomori's trichrome. Sections of pancreas were oxidized with acidified permanganate and stained with aldehyde fuchsin and Gomori's trichrome.

The same nomenclature of pituitary cell types is used in this report as in our earlier communication (Bogdanove and Halmi, 1953).

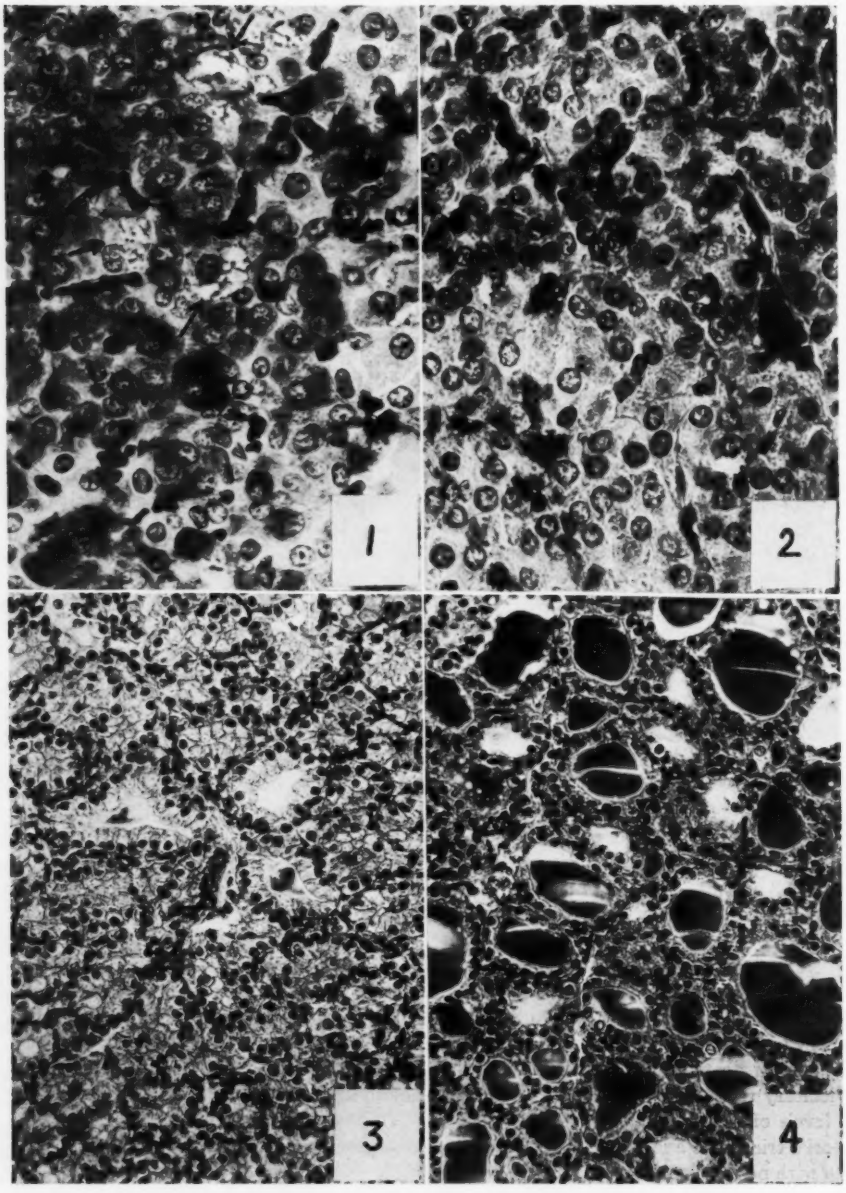

PLATE 1

FIG. 1. Anterior pituitary lobe of lesion-bearing rat which failed to show regression of any endocrine gland and whose thyroid displayed histologic signs of stimulation. Cytoplasm of acidophils appears dark. The arrows point to thyroidectomy cells. Gomori's trichrome stain. 500×.

RESULTS

1. *Alterations of anterior pituitary and target organ structure in rats with hypothalamic lesions (groups I and II)*

Changes of the pituitary acidophils, varying in severity, occurred in 39 of the 56 rats in these two groups. They consisted of decreases in size, number, and density of granulation (compare Figs. 1 and 2) and, in some instances, of cytoplasmic vacuolation. Although these acidophil alterations were not necessarily concomitant with changes in the other chromophils, they were most severe in rats which also exhibited marked regression of the gonadotrophs.

Gonadal atrophy was found in 29 rats. Testicular weights ranged from 324 to 1000 mg. in 19 of these animals, and from 1000 to 2000 mg. in the other 10. In one of the latter rats, whose testes weighed 1400 mg., the accessory sex glands were normal and the histological alterations of the gonads were similar to those characteristic of cryptorchidism. In the other 28, testicular structure resembled that seen after hypophysectomy and was accompanied by accessory sex gland atrophy which was roughly commensurate in degree with that of the testes, the smallest seminal vesicles weighing 61 mg. Among these 28 rats, there were some whose testes displayed a combination of cryptorchid-like and hypophysectomy-like regression.

The gonadotrophs were indistinguishable, or displayed extreme regression, in the pituitaries of the rats with the most severe hypophysectomy-like testicular atrophy. In most of the animals with less severe gonadal regression of this type, the gonadotrophs were decreased in size, number and density of granulation, but in others such changes were not apparent. The hypophyses of intact controls, of lesion-bearing rats with normal testes (weighing over 2500 mg.), and of the rat with purely cryptorchid-like gonad atrophy, contained numerous, well-granulated gonadotrophs and occasional castration cells.

Most of the thyroids of the 56 lesion-bearing animals were comparable in size (10 to 20 mg.) and gross appearance with those of the controls. In 4 rats of group II the thyroids were pale, resembling those of hypophy-

FIG. 2. Anterior pituitary lobe of lesion-bearing rat with atrophy of gonads. Acidophils greatly reduced in size (cf. Fig. 1). The larger pale cells in the lower left part of the field are somewhat enlarged and poorly granulated thyrotrophs. Note normal vascular pattern. Gomori's trichrome stain. 500×.

FIG. 3. Exceptionally active appearing thyroid of a lesion-bearing rat (no goitrogen treatment). Note almost complete absence of colloid and tall columnar follicular epithelium. Gomori's trichrome stain. 250×.

FIG. 4. Representative field from the least active appearing thyroid seen in any lesion-bearing rat of this study (no goitrogen treatment). Most follicles contain dense colloid. The follicular epithelium is cuboidal. Gomori's trichrome stain. 250×.

sectomized rats, and weighed less than 8 mg. (minimum: 5.6 mg. in a 422 gm. rat). However, even these glands displayed cuboidal or somewhat taller follicular cells (Fig. 4). The thyroids of the other 52 rats in groups I and II (as well as those of the unoperated controls) varied somewhat histologically, but their acinar epithelium was never flatter than low columnar. In some of the lesion-bearing animals the thyroids appeared extremely stimulated (Fig. 3) and were almost devoid of colloid. The pituitaries of rats with such active appearing thyroids generally contained numerous thyroidectomy cells (Fig. 1), some of which still had beta granules in their cytoplasm. Normally granulated thyrotrophs were present in all pituitaries, even those in which the acidophils were almost completely degranulated and gonadotrophs were not distinguishable.

The adrenals weighed between 32 and 74 mg. in 52 of the 56 operated rats, and were comparable in appearance to those of the controls. In 4 cases, however, they were distinctly atrophic and weighed as follows: 23.4, 22.8, 15.0, and 12.6 mg. This atrophy was primarily due to shrinkage of the fasciculate and reticular zones of the cortex (compare Figs. 5 and 6), i.e., a change similar to that which follows hypophysectomy. All 4 of these rats also exhibited cuboidal thyroid epithelium, severe gonadal regression, marked degranulation of the acidophils and virtual disappearance of the gonadotrophs. However, all but the first of these changes were also found in a number of rats which did not develop adrenal cortical atrophy.

2. *Changes in intermediate and posterior pituitary lobes.*

Twenty-one of the rats in groups I and II exhibited hyperplastic changes of the intermediate lobe similar to those previously described (Bogdanove and Halmi, 1953). In 22 animals the posterior lobe was shrunken, highly cellular, and devoid of aldehyde fuchsin staining neurosecretory material.

3. *The thyroidal iodide concentrating mechanism in lesion-bearing rats not treated with propylthiouracil*

In 8 rats of group I (killed 34–50 days postoperatively) the thyroid-serum iodide gradients (T/S ratios) averaged 40.0 ± 4.84[4] (range: 12.1–59.1). In the 11 rats of group II this value was 27.8 ± 3.5 (range: 8.3–46.2). Some of the least active appearing thyroids possessed a great ability to concentrate iodide, whereas other glands, which appeared stimulated (by histological criteria), had low T/S ratio values. The mean gradient for normal rats on the same diet has been found to be 25.7 ± 0.6 (Halmi *et al.*, 1953), and that for rats hypophysectomized for 29 days only 2.7 ± 0.10 (Halmi, 1954). Since T/S ratio values as low as 8.3 have been found occasionally in normal rats, and since none of the lesion-bearing animals exhibited gradient values as low as those of hypophysectomized rats, these

[4] Mean ± standard error.

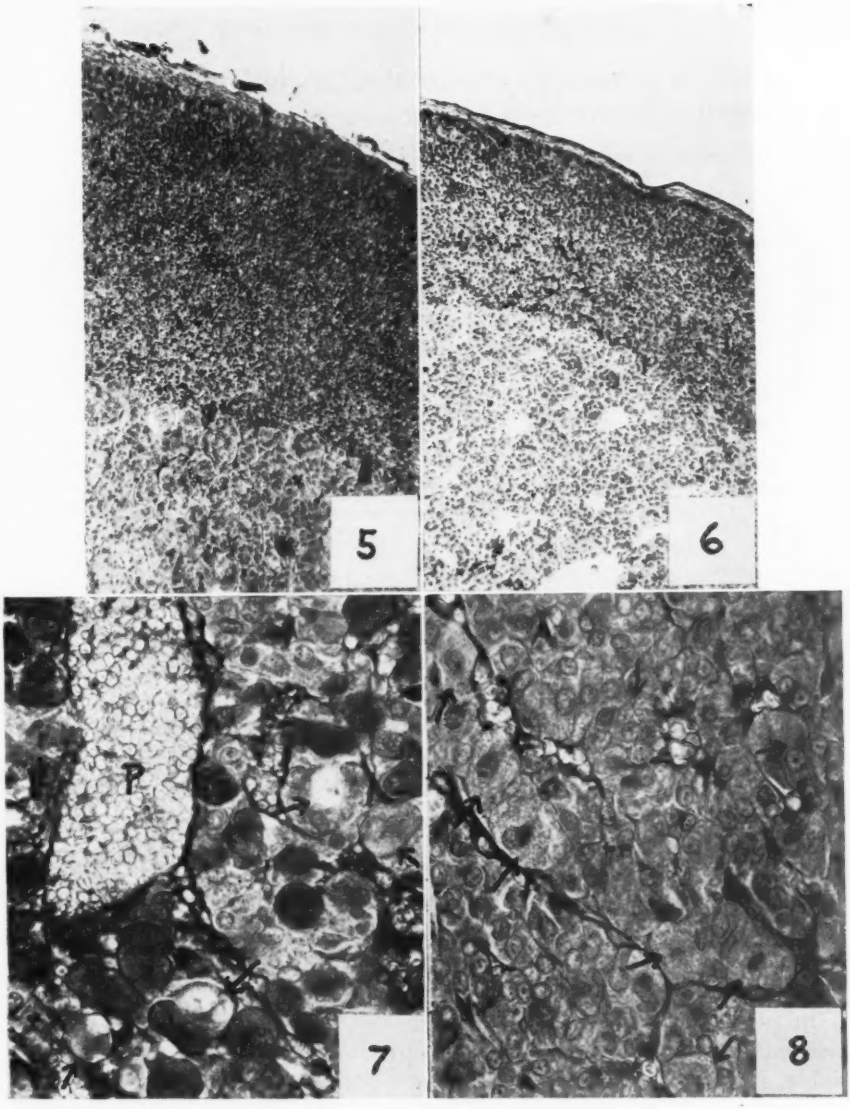

PLATE 2

FIG. 5. Normal adrenal in a lesion-bearing rat. Gomori's trichrome stain. 50×.

FIG. 6. Adrenal of a lesion-bearing rat with marked involution of the cortex, especially the fasciculate and reticular zones (cf. Fig. 5). Medulla appears normal. Gomori's trichrome stain. 50×.

FIG. 7. Anterior pituitary of a rat in which castration and propylthiouracil treatment followed the placement of a hypothalamic lesion that had not led to testicular atrophy. Dark castration cells numerous and prominent. The arrows point to thyroidectomy cells. P = portal vessel. PAS-hemalum. 400×.

FIG. 8. Anterior pituitary of a rat treated as described in the caption to Fig. 7. In this animal the hypothalamic lesion had led to severe testicular atrophy of the hypophysectomy-type. Castration cells are absent, but thyroidectomy cells (arrows) with incipient peripheral vacuolization are numerous. PAS-hemalum. 400×.

results provide no conclusive evidence that thyroidal iodide collection can be depressed by hypothalamic lesions.

4. *Gonadotrophin assays of pituitaries*

The results of the assays are summarized in Table 1. No gonadotrophin could be demonstrated in the half-pituitaries from lesion-bearing rats with severe testicular atrophy. The administration of half-pituitaries from operated rats with little or no testicular atrophy failed to increase ovarian weight in the recipients, but did cause uterine enlargement. Half-pituitaries from intact controls contained sufficient gonadotrophin to produce boht uterine and ovarian enlargement in the test animals. Since uterine weight

TABLE 1. GONADOTROPHIN ASSAYS OF HALF-PITUITARIES FROM RATS WITH HYPOTHALAMIC LESIONS AND FROM UNOPERATED CONTROLS

	Donors			Recipients	
	No. rats	Testicular weights (mg.)	Half-pituitary weights (mg.)	Ovarian weights (mg.)	Uterine weights (mg.)
1. *Recipient controls*	10	—	—	19 ± 1.1* (14–23)†	45 ± 3.8 (28–63)
2. *Unoperated control donors*	16	2886 ± 26 (2404–3249)	4.2 (9)‡ (3.2–5.3)	61 ± 7.0 (21–102)	118 ± 6.0 (84–158)
3. *Operated donors*					
a. Moderate or no testicular atrophy	10	2444 ± 196 (1610–3212)	2.2 (4) (1.4–4.2)	23 ± 1.9 (14–35)	91 ± 13.0 (33–150)
b. Severe testicular atrophy	9	546 ± 32 (416–760)	3.2 (7) (2.0–4.0)	18 ± 0.9 (15–23)	47 ± 2.3 (38–56)

* Mean ± standard error.
† Range.
‡ Number of half-pituitaries weighed.

is the more sensitive of these two indices of gonadotrophin level (Heller *et al.*, 1937), the assay results can be interpreted as indicating that pituitary gonadotrophin content was decreased in all of the lesion-bearing rats, even those whose testes and accessory sex glands appeared normal. Although not every half-pituitary taken for assay was weighed, the majority were. Half-pituitary weights are included in Table 1.

In the histologically examined half-pituitaries of animals with severe testicular atrophy the gonadotrophs were either very sparse or not indentifiable. However, there was no obvious difference between the appearance of these cells in the half-pituitaries from operated rats with well maintained gonads (but diminished stores of gonadotrophic hormone) and in those of intact controls.

5. *Pituitary and thyroid in lesion-bearing rats castrated and injected with propylthiouracil (group III)*

At the time of gonadectomy, hypophysectomy-like testicular atrophy (testis weight <1200 mg.) was evident in 10 of the 17 rats in this group. In the pituitaries of these 10 rats castration changes failed to develop; in fact, gonadotrophs could not even be distinguished in them (Fig. 8). On

the other hand, the characteristic hypertrophic-hyperplastic transformation of the gonadotrophs did occur in the 5 control rats of this group and in 6 lesion-bearing animals with testicular weights greater than 2900 mg. One lesion-bearing rat, whose testes had weighed 2200 mg. at the time of their ablation, is of special interest. In this animal the castration reaction was pronounced in one half of the anterior lobe, whereas the other half lacked gonadotrophs entirely.[5]

The thyrotrophs were transformed into thyroidectomy cells in all the rats of group III, as well as in the 5 controls. This metamorphosis appeared to be independent of the effects of lesions upon the gonadotrophs (Figs. 7 and 8), and was symmetrical in the gland which displayed the striking asymmetry of the castration response. It was not possible to relate the number of thyroidectomy cells to the size of the thyroid, since these cells abounded in every pituitary of group III. However, thyroidectomy cell vacuolation seemed to be more advanced in the animals with the largest thyroids than in those in which goiter formation was somewhat impaired.

The thyroid glands of the 5 controls ranged in weight between 40.8 and 59.2 mg. (mean: 51.9 mg.). They weighed between 19.6 and 49.7 mg. (mean: 34.0 mg.) in the 6 lesion-bearing rats whose testes weighed over 2900 mg., and from 16.0 to 45.2 (mean: 28.9 mg.) in the 10 animals whose gonads were lighter than 1200 mg. The rat with the asymmetric castration response had a thyroid which weighed 36.4 mg. Thyroids heavier than 25 mg. exhibited the hypertrophy and hyperplasia, loss of colloid and hyperemia which are characteristic of PTU treatment. Glands which were smaller than this tended to appear somewhat less stimulated, in that there was more storage of colloid and less vascular dilatation, although the follicular epithelium was as tall as in the larger glands.

It is evident that some of the lesions interfered with the goitrous response to PTU, as observed previously (Greer 1951, 1952; Bogdanove and Halmi, 1953), and that this impedance of goiter formation was not necessarily accompanied by gonad atrophy and abolition of the hypophysial castration response. Conversely, the latter phenomena did occur in some rats whose thyroids were as large as those of the controls.

The adrenals of the lesion-bearing rats in group III weighed between 36.0 and 81.8 mg. and those of the controls between 51.8 and 78.0 mg.

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[5] The lesion was somewhat asymmetrical in this rat and presumably spared the "gonadotrophic" region of the hypothalamus on the side of the normal castration response. If such was the case, the ipsilateral (neurovascular?) hypothalamo-hypophysial link in this particular animal must have failed to cross the midsagittal plane or even to establish functionally significant anastomoses with its counterpart on the other side. Observations on the pattern of the pituitary response to castration in rats with unilateral hypothalamic lesions may reveal whether this was merely an individual peculiarity or whether a unilateral influence of hypothalamic regions on each half of the anterior lobe commonly exists.

6. *Other effects of hypothalamic lesions*

In a number of the rats with hypothalamic lesions hyperphagia (leading to varying degrees of obesity) became evident. The majority of the hyperphagic animals developed testicular atrophy. All of the pancreases examined (which included those of markedly hyperphagic and obese animals) contained apparently normal islets of Langerhans, with well granulated alpha and beta cells.

Some of the rats became savage after the hypothalamic intervention. This change was frequently associated with hyperphagia.

DISCUSSION

Soulairac and Desclaux (1947), who observed degranulation of the acidophils in the hypophyses of rats with hypothalamic lesions, considered this the morphologic manifestation of an outpouring of trophic hormones. The appearance of the altered acidophils in our lesion-bearing rats suggests that their changes were of a regressive nature. Since acidophils are generally accepted to be the source of somatotrophin (STH), one might expect to find physiologic evidence of lessened STH secretion following hypothalamic intervention, as the functional corollary of the morphologic regression of these cells. Two studies tend to support this expectation: 1) Bogdanove and Lipner (1953) have shown that animals with hypothalamic lesions frequently fail to grow, even when they become obese, and apparently deposit adipose tissue at the expense of such protein-rich body constituents as muscle and bone. These observations are consonant with deficient secretion of the protein anabolic hormone, STH. 2) Lesion-bearing rats often exhibit an increased hypoglycemic response to insulin which can be alleviated by the administration of STH (Spirtos *et al.*, 1954). However, neither insulin sensitivity nor growth arrest are invariably associated with discernible changes in the acidophils, and a causal relationship between the morphologic alterations of these cells and the apparent STH deficiency in rats bearing hypothalamic lesions cannot be established on the basis of present evidence.

The gonadal alterations, as well as the corresponding changes of the hypophysial gonadotrophs, were the same in the rats of this study which were not treated with propylthiouracil, as those described, depicted and discussed in our previous paper (Bogdanove and Halmi, 1953). The dearth of gonadotrophin in the pituitaries of rats with severe gonad atrophy of the hypophysectomy-type correlated well with the drastic regression of the gonadotrophs in such animals. The fact that pituitary gonadotrophin content was definitely less than normal in lesion-bearing rats which exhibited little or no testicular atrophy lends itself to two possible interpretations: a) The rate of gonadotrophin secretion may be normally greater than necessary for target organ maintenance.[6] In this case, decreased elabo-

ration of gonadotrophin after the placing of hypothalamic lesions would not be reflected by gonadal atrophy unless titers of circulating hormone fell below a "critical level," whereas concomitant alterations of hypophysial stores would be readily detectable by pituitary gonadotrophin assays. b) Hypothalamic lesions may impair the capacity of the pituitary for storage (without necessarily decreasing the output) of gonadotrophin.

The possibility was also considered that rats with low stores of pituitary gonadotrophin and normal testes might be animals in which circulating hormone titers had not *yet* fallen below the "critical level" at the time of sacrifice. However, *in vivo* palpation of the testes of rats in group II revealed that animals which did not exhibit a reduction of testicular size within 3 weeks failed to develop gonad atrophy even when allowed to live for $2\frac{1}{2}$ to 3 months after placement of the lesions.

The repeated finding of low stores of gonadotrophin in half-pituitaries, the contralateral halves of which appeared to contain a normal concentration of well granulated gonadotrophs, requires interpretation. A certain doubt as to the validity of comparing the structure of one-half of the anterior lobe with the hormone content of the other is naturally suggested by the occurence, in one animal in group III, of an asymmetric histological response to castration. However, the infrequency with which such morphological asymmetry has been found makes it appear improbable that such a phenomenon could have significantly affected the results of these assays. It is possible that, despite an apparently normal *concentration* of gonadotrophs in the hypophyses of lesion-bearing rats with well maintained gonads, differences in the amount of pituitary tissue (and consequently in the *absolute number* of gonadotrophic cells) administered might account for the discrepancy in assayable potency between the half-pituitaries of this group and those from intact controls. Half-pituitary weights in this group were but 52% of those of unoperated animals (2.2 vs. 4.2 mg.). In addition, it may well be that attempts at quantitating the PAS-positive granulation of the gonadotrophs would reveal a morphologic difference between the anterior lobes of these two groups of rats.

In any event, the occurrence of lowered gonadotrophin stores in rats with well maintained testes and accessories, and apparently normal gonadotrophs, indicates that these stores are a more sensitive parameter of gonadotrophin secretion than are morphological changes. It therefore becomes evident that, in studies aimed at anatomically delimiting the hypothalamic gonadotrophic mechanism by means of lesions, pituitary gonadotrophin assays provide the most reliable index of impairment.

The failure of castration changes to develop in the pituitaries of lesion-bearing rats with severe testicular atrophy is in keeping with the interpre-

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[6] This statement is supported by the well-known fact that incomplete hypophysectomy often fails to cause gonadal regression.

tation that in such animals the gonadotrophic mechanism is grievously disturbed. It would be of interest to determine whether any gonadotrophin could be demonstrated in such pituitaries. Barrnett and Greep (1951) observed a similar lack of castration cells in the hypophyses of stalk-sectioned castrates, but did find a slight elevation in the gonadotrophic potency of these glands as compared with those of stalk-sectioned controls. Although unequivocal hypophysial castration changes took place in our castrated lesion-bearing rats whose testes had not undergone atrophy at the time of their ablation, bioassays would be required to demonstrate whether the gonadotrophin content of their pituitaries had actually risen to the same extent as that of castrates without hypothalamic lesions.

Although the thyroids of most of the lesion-bearing rats which received no goitrogen were grossly normal, those of four were unusually small. Even those glands, however, did not display hypophysectomy-like atrophy of the follicular epithelium, and their ability to concentrate iodide was as great as that of the thyroids of unoperated animals. Thyrotrophs, obviously more resistant to the disturbing effects of hypothalamic lesions than either acidophils or gonadotrophs, were present in every pituitary, although many of the hypophyses exhibited hyperplastic changes of the intermediate lobe such as, in our experience, frequently occur in the case of lesions which impair goiter formation in response to propylthiouracil (PTU). Therefore, neither our morphologic nor our physiologic observations reveal any serious curtailment of thyrotrophin (TSH) secretion in rats not treated with PTU, even though in many cases their lesions must have been similar in location to those which impede the excessive secretion of TSH that follows the administration of PTU.

These findings are in agreement with Uotila's (1940) concept that the pituitary is intrinsically able to produce sufficient TSH to maintain a "basal" level of thyroid function, but that intact hypothalamo-hypophysial connections are necessary for enhanced TSH secretion. Further support for this concept comes from the experiments of Greer *et al.* (1953) who found that pituitary grafts, located in the eyes of hypophysectomized mice, produced sufficient TSH markedly to stimulate thyroidal iodine metabolism although they failed to maintain normal thyroid weights. On the other hand, Ganong *et al.* (1954) have observed hypophysectomy-like atrophy and severely diminished thyroidal iodine collection in dogs with circumscribed lesions in the anterior hypothalamus which did not necessarily lead to panhypopituitarism. The precise role of the hypothalamus in the maintenance of the pituitary-thyroid axis therefore remains problematic.[7]

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[7] The exceptionally active appearance of the thyroids in some lesion-bearing rats is of interest. It has been suggested (Halmi, 1952b) that highly stimulated thyroids in rats maintained on high iodine commercial diets such as Rockland pellets indicate the presence of goitrogens in such feeds. Hypothalamic hyperphagia in the absence of

The equally contradictory observations on the role of the hypothalamus in the regulation of the adrenocorticotrophic function of the pituitary have been admirably reviewed by Munson and Briggs (1955). McCann (1953) has found that, in rats, lesions in the median eminence may abolish the fall of adrenal ascorbic acid content in response to stress without, however, causing adrenal cortical atrophy. A similar failure of hypothalamic lesions to produce adrenal cortical atrophy in dogs has been observed by Ganong and Hume (1954). The 4 rats of our series which displayed such atrophy also had atrophic gonads and small thyroids, which suggests panhypopituitarism. Therefore, data from these animals cannot be taken as evidence for the existence of a hypothalamic mechanism specifically concerned with the morphologic maintenance of the adrenal cortex. Recent studies of McCann and Brobeck (1954) on rats showed that adrenal atrophy followed partial interruption of the supraoptico-hypophysial pathway leading to mild diabetes insipidus. Lesions producing severe diabetes insipidus, however, caused adrenal enlargement, even though such rats failed to respond to adrenalectomy with the normal elevation of ACTH output (McCann and Sydnor, 1954).

Barrnett and Greep (1951) considered abolition of the castration response by pituitary stalk-section to be part of a panhypopituitary syndrome caused by interference with the nutritive vascular supply to the gland. In our study a similar abolition of the castration response was found in rats with hypothalamic lesions which had produced testicular atrophy. However, in all of these animals the adrenals were well maintained and PTU treatment resulted in transformation of the thyrotrophs into thyroidectomy cells. In some of them, thyroid enlargement in response to PTU was as great as in animals which had not suffered hypothalamic damage. Conversely, a diminished response of the thyroid to PTU took place in other rats whose gonads had failed to atrophy after placement of the lesions, and whose pituitaries displayed typical castration changes. These findings support our previously expressed view (Bogdanove and Halmi, 1953) that separate (although perhaps anatomically overlapping) hypothalamic mechanisms participate in the regulation of the thyrotrophic and gonadotrophic functions of the adenohypophysis.

SUMMARY

Bilateral hypothalamic lesions were produced electrolytically in adult male rats by means of a stereotaxic apparatus.

The commonest effect of these lesions was a reduction in size, number, and density of granulation of the pituitary acidophils. The next most common change in anterior lobe histology was regression of the gonado-

interference with the production of thyrotrophin could be expected to result in an increased intake of the hypothetical goitrogen(s) and consequent activation of the hypophysial thyrotrophic mechanism.

trophs, accompanied by hypophysectomy-like atrophy of the testes and accessory sex glands. The thyrotrophs were maintained even when the other chromophils were severely reduced in number or had completely disappeared. The histologic appearance of the thyroid was variable, but in no instance was the follicular epithelium lower than cuboidal. Thyroid-serum iodide concentration ratios were within the normal range. Adrenal cortical atrophy was encountered in four cases, but was not associated with any specific changes in pituitary structure. Further sequelae of the lesions were hyperplastic changes of the intermediate pituitary lobe, atrophy of the posterior lobe, obesity and savage behavior.

Gonadotrophin assays were carried out on half-pituitaries from intact rats and from lesion-bearing rats with and without gonad atrophy. No gonadotrophin was detectable in pituitary tissue from animals with severe testicular atrophy. The glands of lesion-bearing rats with little or no testicular atrophy contained significantly less gonadotrophin than those of intact controls.

Lesion-bearing rats with pronounced gonadal regression failed to develop hypophysial castration changes following orchidectomy, whereas the response to castration appeared unimpaired in animals with well maintained testes. Complete abolition of the morphological castration reaction of the pituitary was compatible with the typical transformation of thyrotrophs into thyroidectomy cells, and the formation of goiter, in response to propylthiouracil treatment. Conversely, lesions which impaired the goitrous response to chemical thyroidectomy did not necessarily impede hypophysial castration changes.

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DIETARY REPAIR OF DEFECTIVE LIPOGENESIS AND CHOLESTEROGENESIS (FROM C^{14}-ACETATE) IN THE LIVER OF THE HYPOPHYSECTOMIZED RAT[1]

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EARLIER reports from this laboratory dealt with the conversion of the C^{14} of acetate-1-C^{14} to cholesterol, fatty acids, and CO_2 by liver slices prepared from hypophysectomized rats that had been fed a stock diet adequate in all respects and containing ground whole wheat as the main carbohydrate source (Tomkins *et al.*, 1952; Baruch and Chaikoff, 1955). Under those dietary conditions, the incorporation of the C^{14} into fatty acids and cholesterol was depressed, but that into CO_2 was well within the normal range. Since the availability of glucose is of importance in the maintenance of lipogenesis (Masoro *et al.*, 1950; Lyon *et al.*, 1952; Masri *et al.*, 1952; Hirsch *et al.*, 1954), we have investigated both lipogenesis and cholesterogenesis in the livers of hypophysectomized rats that were fed synthetic diets in which the amounts and type of carbohydrate were controlled. It is shown here that restoration of hepatic lipogenesis and cholesterogenesis to normal can be achieved in the hypophysectomized rat by special dietary means.

EXPERIMENTAL

Treatment of Animals.—Rats of the Long-Evans strain were hypophysectomized via the parapharyngeal route. The bases of the brains of the operated rats were examined at necropsy, and only data from those in which hypophysectomy was complete are recorded in this study. The composition of the synthetic diets is shown in Table 1. The stock diet contained 67.5% ground whole wheat, 15% casein, 7.5% powdered skim milk, 0.75% NaCl, 1.5% calcium carbonate, 6.75% lard, 1% fish oil (vitamins A and D), and a few drops of a KI solution.

Preparation of Tissues and Incubation Procedure.—Blood was withdrawn from the heart just before the animals were sacrificed by cervical fracture. The livers were rapidly excised and placed in cold Krebs-Henseleit bicarbonate buffer (Krebs and Henseleit, 1932). Slices of approximately 0.5 mm. were prepared freehand, with a thin razor blade, and deposited in fresh cold buffer. The slices were gently blotted with filter paper, and 500 ± 5-mg. portions were placed in the main compartment of a 50 ml. incubation flask containing 5.0 ml. of the bicarbonate buffer (pH 7.3–7.4) to which 2 μm. of acetate-1-C^{14} as the sodium salt had been added. The flasks were gassed with a mixture of 95% O_2 and 5% CO_2, capped with a self-sealing rubber stopper, and incubated, with shaking, at 37.5° for 3 hours.

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Analytical Procedures.—At the end of the incubation period, 0.25 ml. of 30% KOH was injected through the stopper into the center well, and, in a similar manner, 0.25 ml. of 5N H_2SO_4 was added to the incubation medium. At least 30 minutes were allowed for absorption of the $C^{14}O_2$, and the contents of the center well were then quantitatively transferred to a volumetric flask. Aliquots of the mixture containing the radioactive $C^{14}O_2$ were precipitated as $BaCO_3$ according to the method of Entenman *et al.* (1949), and their radioactivity was determined with a thin end-window Geiger-Mueller tube. Fatty acid-C^{14} and cholesterol-C^{14} were determined as described elsewhere (Baruch and Chaikoff, 1955; Hotta *et al.*, 1954).

Liver glycogen was isolated according to Sjogren *et al.* (1938) and measured as glucose by the method of Kemp and Kits Van Heijningen (1954). Plasma glucose was determined by the method of Mendel and Hoogland (1950).

Liver fatty acids were isolated in the following manner. An aliquot of the chloroform extract used for determination of fatty acid-C^{14} was taken to dryness on a steam bath

TABLE 1. COMPOSITION OF SYNTHETIC DIETS

Constituent	Diet A	Diet B	Diet C
	Per cent	Per cent	Per cent
Glucose	60	—	25
Corn starch	—	60	—
Casein	22	22	47
Celluflour	9.8	9.8	19.8
Vitamin B mixture*	0.2	0.2	0.2
Liver Viobin	2.0	2.0	2.0
Salt mixture†	6.0	6.0	6.0
Vitamins A and D	‡	‡	‡

* Contained the following components (mg. per kilo of diet): choline chloride, 1000; niacinamide, 50; inositol, 1000; calcium pantothenate, 50; thiamine hydrochloride, 10; riboflavin, 10; pyridoxine, 10; *p*-aminobenzoic acid, 10; biotin, 0.2; folic acid, 2.0.

† Hawk-Oser salt mixture (Hawk *et al.*, 1947).

‡ Vitamins A and D in fish oil were given every third day when the experimental diets were continued for more than three days.

under an atmosphere of CO_2. The residue was re-extracted with petroleum ether and washed five times with water. The petroleum ether-soluble lipids were transferred to a tared flask and taken to dryness with gentle heat and continuous flushing with CO_2. The fatty acid content was then determined gravimetrically. Liver cholesterol was isolated after the manner described by Hotta *et al.* (1954) and measured by the method of Sperry and Webb (1950).

RESULTS

1. *Experiment with Rats Fed Ad Libitum*

The conversion of the C^{14} of acetate-1C^{14} to fatty acids, cholesterol, and CO_2 by slices prepared from livers of hypophysectomized and normal rats fed *ad libitum* is shown in Table 2. For convenience, the results are grouped according to the diet the rats received after hypophysectomy. Those of group A received the stock diet for the first 9 or 10 days, and the 60% glucose diet for the next 3, 7, or 24 days. Those of group B were fed the stock diet for the first 7 or 24 days after hypophysectomy, and 60% starch diet for the next 3 or 7 days. The rats of group C were fed the stock diet for the first 8 days following removal of the pituitary gland, and 25% glucose

TABLE 2. THE INCORPORATION OF C^{14} OF 1-C^{14}-ACETATE INTO FATTY ACIDS AND CHOLESTEROL BY LIVER SLICES FROM NORMAL AND HYPOPHYSECTOMIZED RATS FED VARIOUS DIETS

Each flask contained 500 ±5 mg. tissue and 2 μm 1-C^{14} acetate in 5.0 ml. bicarbonate buffer. Duplicate flanks were incubated for 3 hours at 37.5°, and their contents analyzed separately; average values are reported below.

Group	Rat No.	State	Body wt. gm.	Days diets fed after hypophysectomy or start of experiment				Avge. daily food intake, grams	Sacrificed after hypophysectomy, days	Plasma glucose when sacrificed, mg. %	Liver fatty acids, % wet wt.	Liver glycogen % wet wt.	Liver cholesterol % wet wt.	Per cent added C^{14} recovered as:		
				Stock	60% glucose (A)	60% starch (B)	25% glucose (C)							Fatty acids	Cholesterol	CO_2
A	11	Normal	154		1–3			13		69	2.5	4.4	0.10	37	1.0	11
	12	Normal	196		1–3			13		87	2.9	5.1	0.17	16	2.2	15
	13	Normal	182		1–7			12		109	2.0	6.0	0.09	35	1.7	29
	14	Normal	205		1–7			12		85	2.5	8.8		29	2.4	38
	15	Normal	206		1–24			12		91	2.4		0.13	17	2.4	39
	16	Normal	195		1–24			12		67	2.2	3.6	0.14	31	1.4	27
										Avg.	2.4	5.6	0.13	28	1.9	25
	17	Hyped*	136	1–10	11–13			9	14		2.5	9.1	0.11	23	2.5	14
	18	Hyped	142	1–10	11–13			8	14	59	2.1	6.0	0.16	14	2.1	24
	19	Hyped	144	1–10	11–13			9	14	69	2.6	6.6	0.12	33	2.6	20
	20	Hyped	152	1–10	11–13			8	14	80	3.3	8.0	0.10	22	3.3	
	21	Hyped	162	1–10	11–17			7	18	96	2.2	6.9	0.10	31	2.5	25
	22	Hyped	154	1–10	11–17			9	18	88	3.2	8.0	0.11	30	3.2	24
	23	Hyped	131	1–10	11–17			8	18	112	2.5	8.0	0.09	34	1.1	15
	24	Hyped	131	1–9	10–33			7	34	56	2.4	5.2	0.11	25	2.4	16
	25	Hyped	126	1–9	10–33			8	34	59	2.5	3.4	0.13	29	2.5	24
	26	Hyped	135	1–9	10–33			8	34	58	2.3	3.5	0.14	22	2.3	27
										Avg.	2.6	6.5	0.12	26	2.5	21
B	27	Normal	208			1–3		13		74	3.0	4.5		27		26
	28	Normal	198			1–3		13		89	2.0	3.9		29		31
	29	Normal	201			1–7		14		69	2.7	6.1		19		25
	30	Normal	211			1–7		15		87	2.0	5.2		22		24
										Avg.	2.4	4.9		24		27
	31	Hyped	127	1–7		8–11		7	12	62	3.0	3.1		27		32
	32	Hyped	172	1–7		8–11		8	12	99	2.5	4.9		33		33
	33	Hyped	126	1–7		8–11		9	12	88	2.0	3.6		32		32
	34	Hyped	132	1–24		25–31		7	32	115	2.7	5.1		20		26
	35	Hyped	135	1–25		25–31		6	32	77	2.4	7.3		31		29
	36	Hyped	129	1–25		25–31		8	32	80	2.5	4.2		18		34
										Avg.	2.7	4.7		27		31
C	37	Normal	192				1–15	12		79	1.5	3.2	0.15	3.9	0.53	43
	38	Normal	172				1–15	14		96	1.9	1.2	0.15	5.1	0.76	42
	39	Normal	162				1–15	12		55	1.7	1.1	0.23	3.6	0.63	49
	40	Normal	170				1–15	13		88		1.7	0.13	4.4	0.81	37
	41	Normal	159				1–16	15		79		2.0	0.12	4.0	0.70	41
										Avg.	1.7	1.8	0.15	4.2	0.68	42
	42	Hyped	150	1–8			9–23	10	24	89	1.4	0.9	0.10	1.3	0.16	23
	43	Hyped	145	1–8			9–23	8	24	77	1.7	1.4	0.12	1.0	0.05	53
	44	Hyped	146	1–8			9–23	7	24	101	1.5	1.7	0.21	2.1	0.10	39
	45	Hyped	150	1–8			9–23	7	24	71		0.7	0.14	2.7	0.09	48
	46	Hyped	139	1–8			9–23	7	24	83	1.9	1.4	0.15	1.8	0.20	35
										Avg.	1.6	1.2	0.14	1.8	0.12	40

* Hyped = Hypophysectomized.

diet for the next 15 days. The rats were sacrificed from 12 to 34 days after hypophysectomy. Normal rats that received comparable dietary treatment served as controls for each of the above groups; these rats were fed the stock diet up to the time they were fed diet A, B, or C.

The finding that hepatic lipogenesis was highest in normal rats fed the high glucose or starch diets confirms earlier observations carried out in this laboratory and elsewhere (Masoro *et al.*, 1950; Whitney and Roberts, 1954).

The livers of hypophysectomized rats fed the 25 per cent glucose diet behave, with respect to lipogenesis and cholesterogenesis, exactly like the livers of hypophysectomized rats fed the ground whole wheat diet (Tomkins *et al.*, 1952; Baruch and Chaikoff, in press). Only in the hypophysectomized rats that were fed either the 60% glucose or 60% starch diets did the extent of hepatic lipogenesis and cholesterogenesis reach the levels observed in the corresponding normal controls.

2. *Experiments in Which the Food Intake Was Controlled*

When allowed free access to food, hypophysectomized rats ingest less food than do normal rats of comparable weight (Table 2). This difference in food intake was overcome in Experiment 2 by intubating both normal and operated rats with the same amount of the 60% glucose diet (A). Each rat received by stomach tube, every 4 hours for 3 days before it was sacrificed, 3 cc. of an aqueous slurry containing 1.5 gm. of diet A. In preliminary experiments with normal rats weighing 130 gm. and having free access to diet A, it was observed that the average intake per rat was about 9 gm. in 24 hours. The results of Experiment 2 are shown in Table 3. *It is clear that, even under these experimental conditions, the conversion of the added C^{14} to fatty acids and cholesterol by the livers of hypophysectomized rats is no greater than that observed with livers of normal rats.*

It has been stated that hypophysectomy accelerated the rate of fatty acid synthesis in the liver (Brady *et al.*, 1951; Gurin and Brady, 1953). This view receives no support from the results of the present investigation on the rat.

DISCUSSION

It is shown here that the livers of hypophysectomized rats fed a diet containing 25% glucose do not convert the C^{14} of acetate-1-C^{14} to fatty acids and cholesterol to the same extent as do livers of normal rats fed the same diet. No such difference between normal and hypophysectomized rats was observed when they were fed the 60% glucose or starch diet. These results therefore indicate that the defective lipogenesis and cholesterogenesis observed earlier in hypophysectomized rats fed the whole wheat diet (Tomkins *et al.*, 1952; Baruch and Chaikoff, 1955) was the

result of an unavailability of the carbohydrate in this diet. It has been stated that the carbohydrate constituents of whole wheat are less available for nutritional purposes, presumably because of their inaccessability to digestive enzymes (Hegsted *et al.*, 1954). The findings of Samuels *et al.* are of interest in this connection, for these workers observed high levels of mesenteric fat in hypophysectomized rat provided they were fed daily, by stomach tube, a diet containing large amounts of glucose, sucrose, and dextrin (Samuels *et al.*, 1942).

The results of the present investigation clearly show that when an abundance of glucose is available to the liver, the maintenance of normal

TABLE 3. THE INCORPORATION OF C^{14} OF ACETATE-1-C^{14} INTO FATTY ACIDS AND CHOLESTEROL BY LIVER SLICES PREPARED FROM NORMAL AND HYPOPHYSECTOMIZED RATS FED, BY STOMACH TUBE, IDENTICAL AMOUNTS OF THE 60 PER CENT GLUCOSE DIET (A)

Each flask contained 500 ±5 mg. tissue and 2 μm. acetate-1-C^{14} in 5.0 ml. bicarbonate buffer. Duplicate flasks were incubated for 3 hours at 37.5°, and their contents analyzed separately; average values are reported below.

Rat		State	Plasma glucose	Liver			Per cent added C^{14} recovered as:		
No.	Body wt.			Fatty acids	Glycogen	Cholesterol	Fatty acids	Cholesterol	CO_2
	gm.		mg %	per cent wet wt.	per cent wet wt.	per cent wet wt.			
47	135	Normal	115	2.0	5.6	0.13	27	1.1	30
48	130	Normal	71	2.7	7.0	0.19	30	1.4	28
49	132	Hyped*	85	2.4	4.3	0.16	25	1.2	29
50	130	Hyped*	97	2.3	5.9	0.20	29	1.1	30
51	135	Hyped*	80	1.9	8.2	0.13	28	1.4	27

* Hyped = Hypophysectomized.

hepatic lipogenesis and cholesterogenesis does not require the concurrence of anterior pituitary hormones. The hypophysectomized rat is, however, more sensitive than the normal rat to the "glucose requirements" for lipogenesis and cholesterogenesis. Our results also indicate the need for a careful evaluation of the nutritional state of the hypophysectomized animal before ascribing a metabolic effect to the removal of the anterior pituitary gland.

SUMMARY

1. The incorporation of the C^{14} of acetate-1-C^{14} into fatty acids and cholesterol was compared in liver slices prepared from normal and from hypophysectomized rats.

2. Liver slices prepared from hypophysectomized rats previously fed a diet containing 25% glucose as the sole carbohydrate source lost, to a considerable extent, the capacity for converting acetate carbon to fatty acids and cholesterol.

3. The previous feeding of a diet containing 60% glucose or starch to the hypophysectomized rat restored to normal the capacity of its liver to synthesize cholesterol and fatty acids from acetate.

4. The conclusion is drawn that, provided an abundance of glucose is

available to the rat, the maintenance of normal hepatic lipogenesis and cholesterogenesis is *independent* of anterior pituitary hormones.

5. Our results indicate the need for a careful evaluation of the nutritional state of the hypophysectomized animal before ascribing a metabolic effect to the removal of the anterior pituitary gland.

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STUDY OF THE HYPERGLYCEMIC ACTIVITY OF NORMAL HUMAN URINE[1]

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EARLIER experiments from this laboratory (Moya and Hoffman, 1954) showed that extracts of normal human urine are hyperglycemic and glycogenolytic, and that stimulation of glycogenolysis *in vitro* is due to some agent other than pancreatic glucagon. In later experiments (Moya, 1955) it was shown that the *in vitro* glycogenolytic activity of these preparations was due to the presence of urinary amylase. The present study is concerned with an investigation of the principle in these extracts responsible for their hyperglycemic action. It appears from the findings presented below that the hyperglycemic effect is due to a hypotensive agent which induces the release of endogenous epinephrine, which in turn results in an increase in blood sugar.

METHODS AND RESULTS

Male New Zealand white rabbits weighing between 2 and 4 kg. were used, all animals being fed the same commercial diet (Purina). The substances injected were dissolved in 2 ml. of isotonic phosphate-buffered saline (pH 7.4), prepared as indicated below. *In vitro* incubations were carried out in open test tubes (150 mm. length ×22 mm. inside diameter) shaken at 120 oscillations per minute, air being the gas phase. The incubation medium was prepared by mixing 5 parts of isotonic saline with one part of 0.11 *M* potassium phosphate buffer (pH 7.4). Tissue slices were cut with the aid of a Stadie-Riggs microtome. Glucose was determined by the method of Nelson (1944) using Folin-Wu sugar tubes. To indicate the presence of polysaccharide, hydrolysis of protein-free filtrates was carried out in 0.6 N HCl for two and one-quarter hours in a boiling water bath. Following neutralization the reducing activity of the hydrolysates was determined by the method of Nelson.

Amylolytic activity was determined by a modification of the method of Somogyi (1938). The incubation mixture was as follows: 0.5 ml. 1% glycogen, 0.20 ml. 0.11 *M* phosphate buffer (pH 6.8) and the extract to be tested dissolved in 0.50 ml. saline.

The arterial blood pressure was recorded in the carotid artery, with sodium citrate as anticoagulant. The animals were anesthetized with a mixture of urethane (500–700 mg./kg.) and sodium phenobarbital (130–160 mg./kg.) given intraperitoneally.

Two hyperglycemic urinary extracts have been studied; one was prepared as previously described (Moya and Hoffman, 1954), and will be referred to as APU (the alcohol precipitate from urine); the other was prepared according to the procedure described by Meduna (1948).

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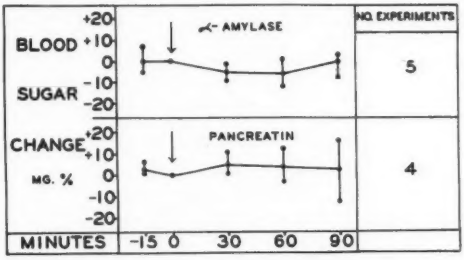

FIG. 1. Blood glucose changes following the intravenous administration of amylase preparations (20 mg./kg.). The arrows indicate the time of injection. Each curve represents the average change in the number of experiments indicated at right. The vertical lines represent the range of change at each point.

Since APU was known to be hyperglycemic and to contain urinary amylase it seemed of interest to test other amylase preparations for hyperglycemic action. The intravenous administration of pancreatin (U.S.P., Merck) or an a-amylase of bacterial origin[2] in doses of 20 mg./kg. of body weight did not affect the blood sugar concentration. The results of these experiments are presented in Figure 1.

It should be recalled that APU in doses of 1 to 5 mg./kg. body weight has consistently been found to increase the blood glucose concentration by 30 to 70 mg.% (Moya, 1955).

The doses of pancreatin and a-amylase used are greater than active doses of APU on the basis of total amylolytic activity. Although the amylolytic activity of APU varied from one preparation to another it was never as high as the activity of pancreatin, and was of the order of activity of a-amylase, as illustrated in Figure 2. Neither APU, pancreatin

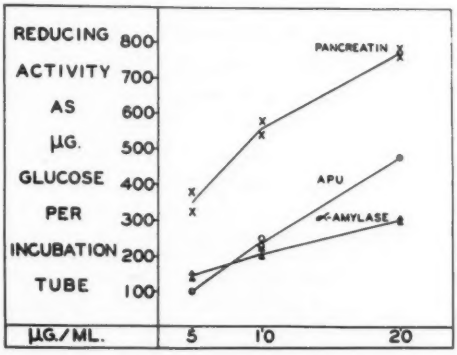

FIG. 2. Amylolytic activity of pancreatin, APU and a-amylase. The reducing activity is expressed as μg. of glucose per incubation tube.

[2] Obtained from Nutritional Biochemicals Co., Cleveland, Ohio.

TABLE 1. EFFECT OF ADDED A-AMYLASE OR PANCREATIN ON THE OUTPUT OF REDUCING SUBSTANCES BY LIVER SLICES *in vitro*

Experiment	Treatment	Dose, μg./ml.	Output of reducing matter as mg. glucose per gm. liver	Average	Increase over control
1	Control	—	8.9, 7.9, 11.0	9.3	—
	APU	100	18.6, 18.7, 20.8	19.4	10.1
	a-Amylase	100	16.7, 16.5, 18.3	17.2	7.9
2	Control	—	14.3, 13.3, 13.7	13.7	—
	APU	100	17.8, 16.3, 16.0	16.7	3.0
	Pancreatin	100	16.0, 15.1, 17.5	16.2	2.5

or the a-amylase used in these experiments contained detectable maltase activity under the conditions of the amylolytic assay presented above.

Previously it had been tentatively concluded that APU-induced hyperglycemia could be due to the presence of urinary amylase (Moya, 1955). Since other amylases were not hyperglycemic, it now appeared that either the urinary enzyme was somewhat unique in this respect or else some other component of the urinary extract was responsible for the hyperglycemic action. As a preliminary investigation it was decided to test the action of pancreatin and a-amylase on liver slices. The results of representative experiments are presented in Table 1.

It can be seen that both enxyme preparations had marked glycogenolytic action, increasing the concentration of reducing matter in the medium to values of the order of those found with equal doses of APU. Since neither of these two preparations is hyperglycemic it became apparent that APU-induced hyperglycemia was not necessarily related to its glycogenolytic activity *in vitro*.

Following acid hydrolysis in 0.6 N HC1, the reducing activity of filtrates was increased, not only in the case of filtrates derived from the incubation fluid of slices treated with amylolytic preparations, but also in the case of untreated control slices. These results appear in Table 2. Although this type of evidence is not conclusive, it suggests that glycogen leaches out of the cells into the incubation fluid and is therefore readily available to the action of added amylolytic enzymes.

The possibility was then considered that the hyperglycemia produced by

TABLE 2. OUTPUT OF REDUCING MATTER BY LIVER SLICES BEFORE AND AFTER ACID HYDROLYSIS OF PROTEIN-FREE FILTRATE

Treatment	Dose, μg./ml.	Reducing activity as mg. glucose per gm. tissue*		
		Before hydrolysis	After hydrolysis	Increase
Control	—	15.0	17.4	2.4
APU	100	18.3	23.9	5.6
Pancreatin	100	22.1	30.5	8.4

* Average of triplicate determinations.

APU was mediated either by epinephrine or an epinephrine-like substance present in the urinary extract, or else by endogenous epinephrine released under the influence of APU. To investigate this possibility it was decided to study the action of the extract on the blood pressure of the rabbit. Figure 3 (A) shows that following the intravenous administration of APU the arterial blood pressure is markedly decreased. It appeared from these

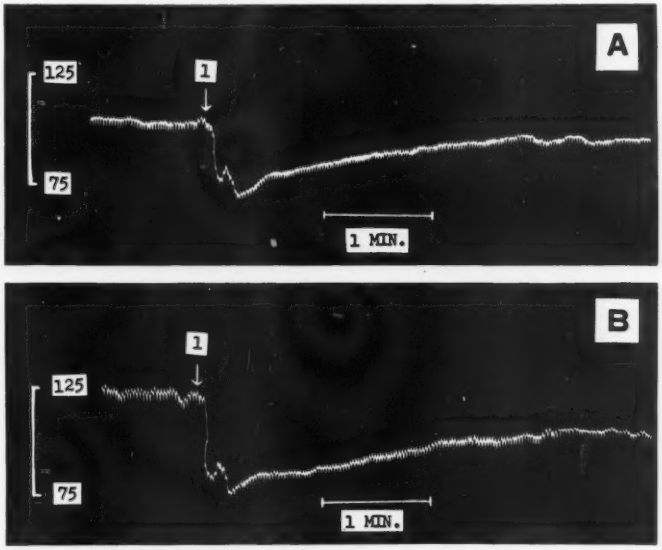

FIG. 3. Tracings showing blood pressure changes in a rabbit (2.7 kg.) following the administration of urinary extracts. Blood pressure in carotid artery in mm. Hg. Extracts given in 0.5 ml. saline through canula in femoral vein. (A) At (1) 74 μg. APU/kg. (B) At (1) 222 μg. Meduna's extract/kg.

experiments that APU contains a strong hypotensive factor and that the hyperglycemic action could be the result of increased secretion of epinephrine following the drop in blood pressure.

Phatak and David (1953) have shown that the adrenolytic drug, *Hydergin*,[3] abolishes the hyperglycemic reaction in the rabbit following the administration of morphine or l-isomethadone. It was decided therefore to test the action of *Hydergin* on APU-induced hyperglycemia. In each experiment one of two rabbits was given 0.1 mg. *Hydergin*/kg. body weight intravenously and 30 minutes later both animals received the same dose of APU. Specimens for blood glucose determinations were taken at the times indicated in Figure 4, from which it can be seen that the adrenolytic agent blocked the hyperglycemic response following APU. Three or

[3] *Hydergin* (Sandoz) is a mixture of equal parts of the methanesulphonate salts of dihydroergocornine, dihydroergocristine and dihydroergokryptine.

four days later the animal which had not responded to APU was treated again with the urinary extract in the absence of *Hydergin*. Curve III in Figure 4 shows that in the absence of *Hydergin* the rabbit responded to APU by the usual hyperglycemic change.

These experiments indicated that hyperglycemia following APU is due to increased endogenous epinephrine secretion resulting from the action of a hypotensive factor in the extract.

Extracts of normal human urine prepared by the method of Meduna (1948) have been found to contain amylase. When administered intra-

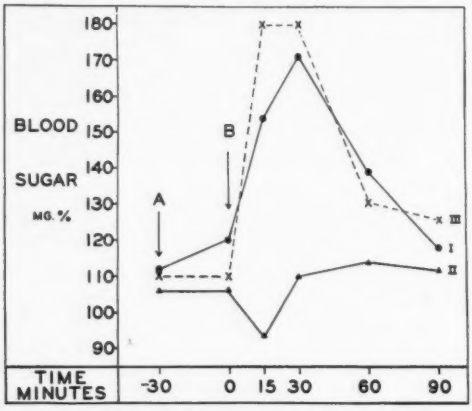

FIG. 4. Blood glucose concentration in the rabbit following the administration of APU in the presence or absence of *Hydergin*.

Curve I: At (A) rabbit received 1 ml. water/kg., and at (B) 5 mg. APU/kg.

Curve II: At (A) rabbit received 0.1 mg. *Hydergin* in 1 ml. water/kg., and at (B) 5 mg. APU/kg.

Curve III: Same rabbit as in Curve II but three days later; at (B) received 5 mg. APU/kg.

All injections given intravenously.

venously to rabbits the extracts cause hyperglycemia which can be inhibited by previous administration of *Hydergin* (Fig. 5). Animals which do not respond to Meduna's extract[4] in the presence of *Hydergin* respond to the extract in the absence of the adrenolytic agent, as shown by Curve III in Figure 5.

DISCUSSION

The material precipitated from acidified normal urine by the addition of two volumes of ethanol (APU) has previously been shown to stimulate *in vitro* glycogenolysis by virtue of the presence of urinary amylase. Wilson and Strieck (1932) found that the administration of crude preparations of pancreatin induced hypoglycemia; however, purified preparations

[4] Meduna's extract, like APU, contains a hypotensive agent as illustrated by Figure 3B.

had no effect on the blood glucose concentration. Similar negative results are presented above and, together with the evidence of increased production of reducing matter by slices incubated with a-amylase or pancreatin, suggest that urinary amylase like other members of the amylolytic family of enzymes is not hyperglycemic. The increase in reducing matter in the medium following the *in vitro* incubation of liver slices in the presence of APU is possibly due to the amylolytic action of urinary amylase, previously found in APU, acting on glycogen that has leached out of the cells into the medium.

From the results presented in this communication it appears that the hyperglycemic action following the intravenous administration of APU or Meduna's extract is an effect secondary to the hypotensive response, and

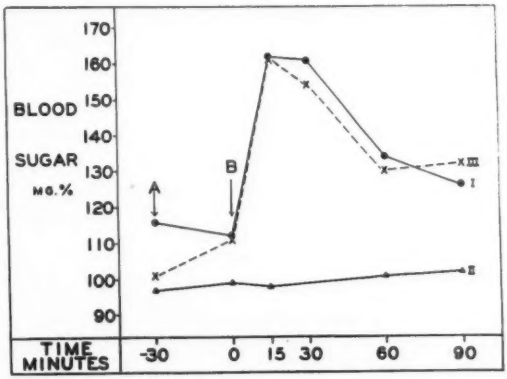

FIG. 5. Blood glucose concentration in the rabbit following the administration of Meduna's extract in the presence or absence of *Hydergin*.

Curve I: At (A) rabbit received 1 ml. water/kg., and at (B) 10 mg. extract/kg.

Curve II: At (A) rabbit received 0.1 mg. *Hydergin* in 1 ml. water/kg., and at (B) 10 mg. extract/kg.

Curve III: Same rabbit as in Curve II but four days later; at (B) received 10 mg. extract/kg.

All injections given intravenously.

probably mediated by endogenous epinephrine. It is not known as yet whether the same substance is responsible for the hypotensive properties of both of these preparations.

Since the active principle in APU is non-dialyzable it appears likely that it be either a protein or bound to a protein. The possibility that kallikrein (Frey, Kraut and Werle, 1950) is the factor responsible for the hypotensive property of these preparations is now being investigated.

SUMMARY

1. The intravenous administration of pancreatin or an a-amylase of bacterial origin was found not to affect the blood sugar of the rabbit.

2. Pancreatin and a-amylase induced an increase in the amount of reducing matter released into the medium by rabbit liver slices incubated *in vitro*.

3. A urinary extract previously found to be hyperglycemic and glycogenolytic appears to stimulate *in vitro* glycogenolysis, not through an intracellular mechanism but by the action of urinary amylase on glycogen that has escaped from the cells into the medium.

4. The urinary extract has been shown to decrease the blood pressure of the rabbit.

5. Hyperglycemia following the administration of the urinary extract can be completely inhibited by the adrenolytic drug *Hydergin*.

6. Extracts of normal human urine prepared by the method of Meduna have been shown to be hyperglycemic when the material is administered intravenously. These extracts also are hypotensive and their hyperglycemic action is inhibited by *Hydergin*.

Acknowledgments

The author wishes to thank Dr. J. C. Szerb for his aid in setting up the blood pressure experiments and Dr. A. K. Reynolds for some of the equipment and materials used in these experiments, both from the Department of Pharmacology, Dalhousie University. The technical assistance of Misses Joan Dewar and Margaret MacIntosh is gratefully acknowledged.

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INHIBITION OF THE RELEASE OF PITUITARY OVULATORY HORMONE IN THE RAT BY MORPHINE[1]

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AMENORRHEA and sterility are frequent accompaniments of morphine addiction in the human female (Menninger-Lerchenthal, 1934; Pescor, 1938). The mechanisms by which these effects are induced, however, are not clearly understood. In experimental animals results are contradictory. Myers and Flynn (1928, 1931) failed to observe any disturbance of estrous cycles, ovulation or fertility despite 132 days of chronic morphine treatment in rats, and their results were confirmed by Forster (1928). In contrast, treatment of mice daily for a two-month period with morphine has been reported to suppress estrus and induce atrophy of the ovary and uterus (Ko, 1934). Similar observations were noted in the rabbit (Bun, 1937).

The finding by Everett and Sawyer (1950) that "spontaneous" ovulation in the rat involves a neurogenic timing factor with a 24-hour rhythmicity offers a new approach to the study of the control of ovulation. These investigators noted in their early experiments that the ovulatory discharge of pituitary gonadotrophin (abbreviated "LH" with reservations) depends upon a neurogenic stimulation of the hypophysis and that the property of spontaneity probably resides in the hypothalamus (Sawyer *et al.*, 1949; Everett *et al.*, 1949). By the use of neural blocking agents such as atropine, dibenamine and nembutal, the studies demonstrated that the critical period at which this neurogenic stimulus occurs is between 2–4 P.M. on the day of proestrus, and that a 24-hour rhythmicity is inherent in the phenomenon (Everett *et al.*, 1949; Everett and Sawyer, 1950).

Since previous reports on the effects of morphine were contradictory, the current investigation was undertaken to re-evaluate the evidence on this problem in rats, giving due consideration to the neurogenic timing mechanism. The results reveal that: (1) ovulation can be blocked by injection of morphine sulfate between 12–2 P.M. of the day of proestrus, (2) treatment after 4 P.M. is ineffective in blocking ovulation, (3) the 24-hour rhythmicity in the timing mechanism is confirmed, and (4) chronic treatment with a given dose of morphine daily before 2 P.M. is capable of block-

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ing ovulation continually for periods up to 25 days. The results incidentally confirm, with a different breed of rat, the finding of the Duke University experiments that the critical period of neurogenic stimulation of the hypophysis is between 2–4 P.M. of the day of proestrus.

The results are of particular interest since the spontaneity of the rat ovarian cycle is somewhat analogous to that of the human being. This is in contrast to conditions in the cat, rabbit, etc., where copulation is necessary to induce ovulation. The results have been reported in abstract (Barraclough and Sawyer, 1954).

MATERIALS AND METHODS

Sexually mature female albino rats of the Sprague-Dawley strain, used in the study, were maintained in a temperature-controlled, artificially illuminated room. Lighting was controlled by a time switch allowing 14 hours of light and 10 hours of dark each day. The rats were purchased at a weight of approximately 200 gm. and kept under these lighting conditions about 30 days before use. Prior to experimentation daily vaginal smears were taken for a 3-week period to insure cyclic regularity. Under these conditions the rats used in this investigation were found normally to run 5-day cycles.

To determine the effective dosage, two levels of morphine sulfate were employed, 50 mg./kg. and 20 mg./kg. The drug was dissolved in Ringer solution and injected subcutaneously at various times during the day of proestrus. Since the critical period of neurogenic stimulation in Sprague-Dawley rats was unknown at the beginning of the present experiments, one group of animals was treated between 12–2 P.M. and another group at 4 P.M. on the day of proestrus. This permits a comparison of the time relationships of the sensitive period in the Sprague-Dawley rats used in our experiments and the Vanderbilt strain employed at Duke University.

A third group of rats each received 50 mg./kg. of morphine sulfate between 12–2 P.M. of proestrus, but they were not autopsied until the second morning after treatment. Autopsy was performed on the two other groups the first morning after sacrificing the animals with ether. At autopsy the oviducts were excised and the ampullae examined under a compound microscope for tubal ova (Everett, 1947). The condition of the uteri and ovaries was also noted as previously described (Everett *et al.*, 1949). Routinely, one ovary was fixed in Susa fixative, serially sectioned and stained by a modified Mallory tri-acid technique (Everett, 1943).

Another series of rats was injected daily for varying periods (13, 25, 30, 38, 49, 52 days) between 12–2 P.M. with 50 ml./kg. of morphine sulfate, beginning in each instance with proestrus. The Fallopian tubes and ovaries were examined for evidence of ovulation in the usual manner.

RESULTS

The physiological reaction of rats to high doses of morphine, as reviewed by Eddy in Krueger *et al.* (1941), occurs in at least 2 stages. In the present experiments, within a half hour after administration of either 20 mg./kg. or 50 mg./kg., there was observed a tendency toward somnolence, depressed reflexes, muscular incoordination and catatonia which was more apparent in the rats receiving the higher dosage. After an hour or more there was a reversal, again more marked with the 50 mg./kg. dosage, in

the direction of an increased muscle tone characterized by rigid extension of the tail. Exophthalmos and salivation were also occasionally observed. Subsequently some of the rats which received the higher dosage of morphine displayed spontaneous clonic convulsions. Signs of recovery appeared 2–3 hours after injection of the 20 mg./kg. dose and 4–5 hours after 50 mg./kg.

Acute morphine studies. Working on the assumption that the critical period of neurogenic stimulation exists between 2–4 P.M., the hours between 12–2 P.M. of the day of proestrus were chosen for injection of morphine sulfate. The results are summarized in Table 1. Injection of 50 mg./kg. of morphine sulfate between these hours prevented ovulation

TABLE 1. EFFECT OF MORPHINE ON OVULATION IN THE RAT

Morphine sulfate dosage mg./kg. s.c.	Time of injection at proestrus (day one)	No. of animals	Results at autopsy on day 2	
			No. ovulated	% blocked
50	12–2 P.M.	16	3	81
	4 P.M.	10	10	0
20	12–1 P.M.	5	5	0
	2 P.M.	4	1	75
			Results at autopsy on day 3	
50	12–2 P.M.	5	5	0

from occurring that night in 13 of 16 animals (81%). If, however, the treatment was delayed until 4 P.M., 100% of the animals ovulated. When a lower dosage of morphine was employed (20 mg./kg.) it was necessary to inject the animals at 2 P.M. to effectively block ovulation; injection of this dosage before 1 P.M. failed to interrupt the process.

To determine whether animals that failed to ovulate on the night following treatment would ovulate the next night, a third group of rats was injected with 50 mg./kg. of morphine sulfate between 12–2 P.M. of proestrus but were not autopsied until 48 hours after treatment. Under these conditions 100% of the animals ovulated the second night (Table 1).

In the animals that failed to ovulate the ampullae of the oviducts were not distended nor were any tubal ova discernible upon autopsy. The uteri remained greatly distended and hyperemic. Histological examination of the ovaries failed to reveal any newly-formed corpora lutea although vesicular follicles were present.

Chronic morphine studies. Since, as demonstrated above, morphine is capable of blocking ovulation when injected between 12–2 P.M. of the day of proestrus, it became of interest to determine whether this drug would continue to be effective in blocking ovulation if administered daily over a prolonged period of time.

A series of rats was injected daily for varying periods (13, 25, 30, 38, 49, 52 days) between 12–2 P.M. with 50 mg./kg. of morphine sulfate and autopsied at the time of a vaginal estrus. All animals ran irregular estrus cycles; the animal treated longest (52 days) exhibited only 5 vaginal estrous smears (Table 2). As is shown in Table 2 and Figure 1B, C, chronic morphine treatment was effective in preventing ovulation for periods up to 25 days, in 3 of 4 animals. If, however, injections were continued beyond this time (30–52 days) ovulation did occur. It is interesting to note that vaginal estrus did not correspond to the time of ovulation in the latter group, since no ova were discernible in the Fallopian tubes at autopsy. Histological examination of the ovaries, however, showed that ovulation

TABLE 2. EFFECT OF CHRONIC MORPHINE ON OVULATION IN THE RAT

Days of treatment	Number of animals	Number of vaginal estrous periods per animal during treatment	Ovarian histology at autopsy
13	1	1	No corpora lutea
25	3	2	No corpora lutea
25	1	2	Corpora lutea present
30	4	2	Corpora lutea present
38	2	3	Corpora lutea present
49	2	4	Corpora lutea present
52	2	5	Corpora lutea present

had occurred in all of the animals injected for more than 30 days, since the corpora lutea present must have been formed during treatment (Fig. 1D).

It was noted also that a rapid loss of body weight occurred in the chronically treated rats soon after initiation of treatment. It became necessary, therefore, to ascertain whether the cessation of estrous cycles and inhibition of ovulation in these morphine-injected animals might be the results of such body weight loss rather than of a specific action of the morphine. A series of 6 normal rats was pair-fed to the 8 experimental animals and daily body weights, as well as vaginal smears, were recorded. As is shown in Figure 2, the rapid loss of body weight, which occurred after initiation of treatment, was paralleled in the pair-fed animals. As morphine injections were continued body weight was recovered and reached preinjection levels about 40 days after initiation of treatment. In contrast to the morphine-treated rats, however, all pair-fed control rats remained cyclic and ovulated normally.

Morphine was withdrawn from 4 rats after 30 consecutive days of treatment, and the animals were than observed for restoration of vaginal cycles, body weight and ability to ovulate with the corresponding vaginal estrus. Three of these animals were autopsied at the second vaginal estrus following withdrawal of morphine (48–52 days after initiation of treatment). While cyclic regularity failed to return at this time, body weight recovery proceeded more rapidly than during treatment. At autopsy some 7–9

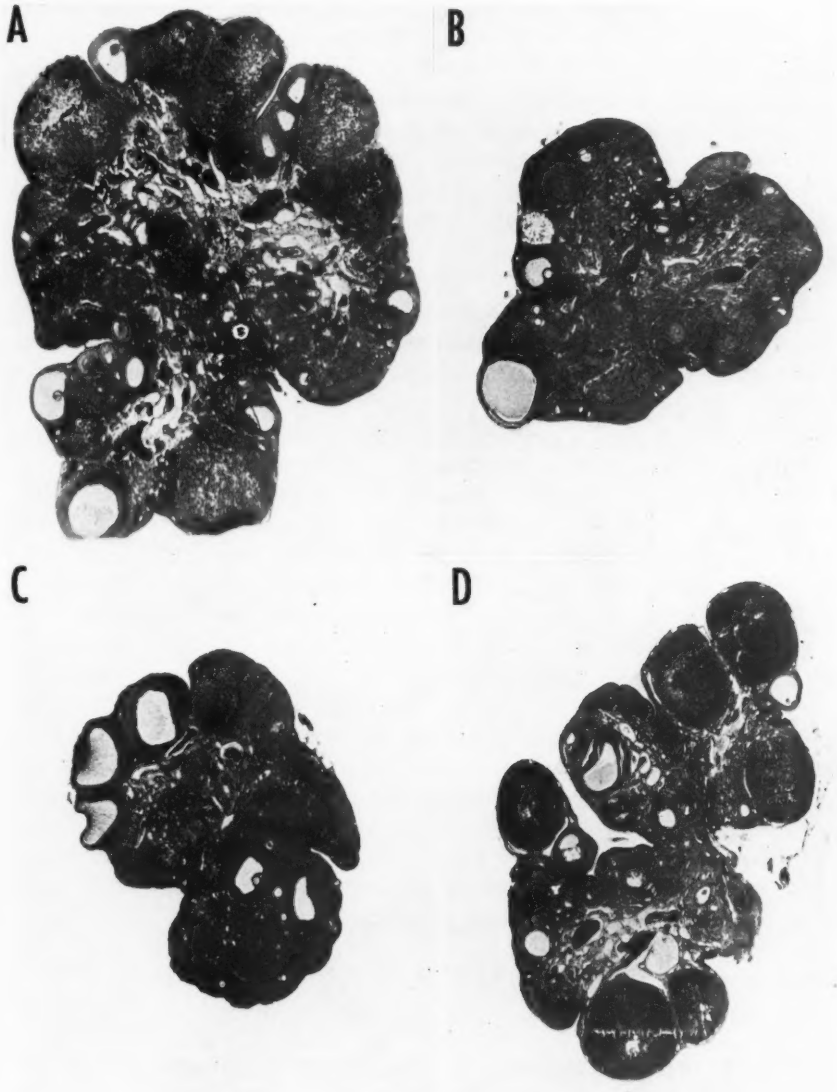

FIG. 1. The effects on ovarian histology of repeatedly blocking hypophyseal activation with 50 mg./kg. morphine sulfate daily. Fig. 1A shows a normal ovary at estrus with several newly-formed corpora lutea. Fig. 1B illustrates conditions after daily treatment with morphine for 13 days. Although autopsy was performed at vaginal estrus there are no new corpora lutea. Fig. 1C shows the reduced ovarian size and absence of newly-formed corpora lutea after 25 daily injections with morphine. Fig. 1D is an ovary from a rat which received morphine daily for 30 days. The corpora lutea appear to have been formed as a result of ovulation during the last 5 days of treatment.

tubal ova were discernible in each case, and histological examination of the ovaries revealed newly formed corpora lutea.

The remaining rat was followed for 70 days after withdrawal of morphine (100 days after initiation of treatment). As in the previous group, body weight recovery proceeded more rapidly after withdrawal than during treatment. Cyclic activity eventually returned 43 days after cessation of treatment (73 days after initiation of treatment) and on autopsy at estrus on the 100th day 9 tubal ova were present.

DISCUSSION

The ability of morphine to block ovulation in the rat furnishes additional evidence that neurogenic stimulation of the hypophysis is essential for the ovulatory discharge of LH. The morphine data confirm in almost every

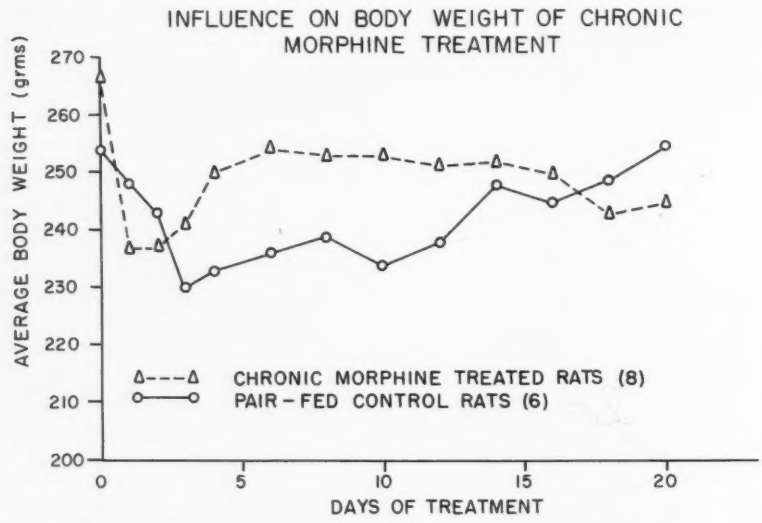

FIG. 2. Effect of chronic morphine treatment and pair feeding on body weight in the rat.

detail the earlier findings concerning the neurogenic timing factor and its diurnal rhythmicity in the control of rat pituitary gonadotrophin.

Such confirmation gains increased significance when one considers that different strains of rats, raised under different conditions in different geographic locations were employed in these studies. Our Sprague-Dawley rats were obtained from Chicago in an adult condition and maintained in California under controlled lighting conditions only a month prior to treatment. The earlier studies in North Carolina used the Vanderbilt strain of rats that had been inbred for many generations under controlled

lighting. Identical results under these widely different conditions emphasize that the neurogenic timing factor is no chance accomplishment of a single strain of inbred rats, but probably a very widespread phenomenon. Controlled animal room lighting would appear to be the most important single requirement in "setting" the timing mechanism, which becomes established within a month of controlled illumination.

Several factors may account for the failure of Myers and Flynn (1928), Myers (1931) and Forster (1928) to observe effects of chronic treatment with morphine on sexual phenomena or body weight in the rat. In the first place their animals were much younger (150 gm.) than ours (250 gm.) at the beginning of treatment and perhaps younger rats are more tolerant of morphine. Their dosages, except in the cases of 2 individual rats of Myers' (1931) series were under 50 mg./kg. Also, it seems likely that their injections may have been made too early or too late in the day to affect the 2–4 P.M. timing mechanism, which was then unknown. In the present investigation the 3 rats that ovulated in spite of treatment with morphine (50 mg./kg.), which blocked 81% of the cases, may have been insensitive to the blocking action of the drug, or the effects of the latter may have dissipated before 4 P.M. Another interesting possibility for speculation is suggested by the observation that one of these rats underwent marked convulsive seizure activity during morphine treatment. Might it be that after morphine blockade of the natural mechanism the seizure activity stimulated the release of pituitary gonadotrophin as it is known to do in the case of pictrotoxin in the rabbit?

That morphine can influence ovulation and vaginal cycles is further substantiated in the results of rats receiving chronic treatment with morphine daily before 2 P.M. Whereas blockade of ovulation was at first complete and later partial, the effect on vaginal estrous periods was reasonably constant over the 7- or 8-week period under observation. The estrous periods were about 12 days apart but, in the absence of corpora lutea, could not have been pseudopregnancy cycles. They are reminiscent of the results reported by Richter (1933) on cutting the pituitary stalk in the rat. Chronic treatment with morphine would apper to be a temporary pharmacological lesion not unlike stalk section in its effects on gonadotrophic secretion.

Morphine also can influence other anterior pituitary functions which are under nervous control, as evidenced by its ability to suppress the release of of adrenocorticotrophic hormone (ACTH). Briggs and Munson (1954a, b) have shown that morphine, administered to rats under pentobarbital anesthesia, blocks the discharge of ACTH following a variety of stresses. The mechanism by which morphine exerts its action in blocking release of pituitary hormones appears to be inherent in its effects on central nervous function, as will be discussed in the following paper (Sawyer, Critchlow and Barraclough, 1955).

The possibility has been suggested that the amenorrhea and sterility observed in the human female morphine addict are attributable to her generally poor physical condition as evidenced by weight loss (Krueger *et al.*, 1941). However, the present results in the rat on chronic treatment indicate that upset reproductive activity is not referable to general inanition: pair feeding of normal rats to experimental animals revealed that the resultant loss of body weight did not interfere with normal cyclic activity or ovulation. Naturally it is impossible to apply results obtained in the rat to the human. However, certain similarities in their sex cycles and in their responses to morphine suggest the working hypothesis that morphine may also affect human reproductive phenomena by blocking neurogenic stimulation of the adenohypophysis. Investigations along these lines in humans have been initiated (Eisenman *et al.*, 1954).

SUMMARY

The effects of morphine on certain reproductive phenomena of the female rat have been investigated. The experimental design utilized the Duke University findings that under controlled lighting conditions in this "spontaneously" ovulating form, neurogenic stimulation of the release of pituitary ovulating hormone occurs between 2–4 P.M. of the day of proestrus.

When mature cyclic female Sprague-Dawley rats were injected with morphine prior to 2 P.M. on the day of proestrus, ovulation was blocked in 13 of 16 cases, but if treatment was delayed until 4 P.M. ovulation occurred in 10 of 10 rats. Rats which failed to ovulate the first night did ovulate the next night if not further treatment was given. Daily administration of the drug prior to 2 P.M. blocked the mechanism for periods up to 25 days after which new corpora lutea indicated a "break through" of the ovulatory mechanism even though normal vaginal cycles had not been restored. That the weight loss in chronically-treated rats is not the important factor in preventing ovulation is indicated by the observation that normal animals, pair-fed to morphine-treated rats, retained cycle regularity and ovulated normally.

The results (1) suggest that morphine-induced sterility may be explained as a sequel to blockade of the release of pituitary gonadotrophin, (2) indicate that the blockade may be exerted on some component of the neurogenic timing factor which normally stimulates the hypophysis, (3) confirm the timing limits of stimulation discovered earlier with other nerve blocking agents on a different strain of rats in another geographic location, (4) are consistent with the concept of 24-hour rhythmicity in the mechanism, (5) suggest that earlier failures to demonstrate an effect of morphine on reproduction in the rodent were in part attributable to unfortunate choices of injection times.

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THE DEIODINATION OF THYROXINE TO TRIIODOTHYRONINE BY KIDNEY SLICES OF RATS WITH VARYING THYROID FUNCTION[1]

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THE identification of triiodothyronine in biological materials and subsequent demonstration of its metabolic activity has stimulated interest in its relationship to thyroxine. Several observations from *in vivo* experiments have led to the concept that thyroxine is converted to triiodothyronine in peripheral tissues (Gross and Leblond, 1951; Gross and Pitt-Rivers, 1952 a; Flock and Bollman, 1954; Kalant, Lee and Sellers, 1955). Further support for this relationship was afforded by the demonstration of the deiodination of thyroxine to triiodothyronine *in vitro* by rat kidney slices (Albright, Larson and Tust, 1954).

The deiodination reaction assumes greater significance if it can be shown to depend upon the level of thyroid activity. The purpose of the present paper is to report experiments on thyroxine deiodination by kidney slices of rats with varying thyroid function.

METHODS AND MATERIALS

Female rats of the Sprague-Dawley strain weighing 100–150 gm. were used. All rats were fed Purina Fox Chow which assured an adequate iodine intake (at least 10 micrograms/rat/day). The normal control group was maintained in individual cages in the animal stock room. One group was made hyperthyroid by exposure to cold (−5 to −10° C.)[3] in individual wire floored cages without bedding for 14 to 16 days (Cramer, 1920). Others were made hyperthyroid by feeding 2% desiccated thyroid in the diet for 18 days. A third group was made hypothyroid by feeding thiouracil, 0.3% in the diet and 0.1% in the drinking water for 28 days. A fourth group of rats was thyroidectomized. In order to obtain enough kidney tissue it was necessary to use two rats for each experi-

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[3] Rats exposed to these conditions show the following evidences of hyperthyroidism: (1)increased height of acinar cells, (2) increased basal metabolic rate, (3) increased levels of TSH in body fluids and urine, (4) increased uptake as well as rate of release of I^{131} from the thyroid gland, (5) failure to survive if maintained on 0.1% thiouracil.

ment. The animals were sacrificed by decapitation, the kidneys aseptically removed and cut into thin slices, which were pooled to eliminate differences in individual animals. Slices representing one-half of a kidney were placed in Warburg flasks containing 3 ml. of Krebs-Ringer phosphate solution pH 7.4. One hundredth of a microgram of chromatographically pure I^{131}-labeled thyroxine[4] was added to each flask. The slices were incubated at 37° C. for periods of 0, 3, 6, 9, 12 and in some instances 24 hours.

As a control for each experiment tissue which had been boiled for 5 minutes was incubated 24 hours. Controls for the thiouracil treated animals were: (1) Kidney slices from rats which were fed a ration containing 0.4% thiouracil for 4 days, a period too short to produce hypothyroidism; (2) Kidney slices from normal rats which were incubated in media containing 3.1×10^{-6} and 3.1×10^{-7} molar concentrations of thiouracil. In a separate series of control experiments with normal kidney slices 0.01, 0.1 and 1.0 microgram of triiodothyronine[5] was added to the reaction media. In another control 1.0 microgram of triiodothyronine and a tracer quantity of radioiodide in place of radiothyroxine were added. In both of these control studies the period of incubation was 9 hours.

At the end of incubation the slices were removed from the media, rinsed, and homogenized in 2 ml. of water. One hundred micrograms of carrier thyroxine[6] and triiodothyronine were added to the homogenate which was then extracted twice with ether. The ether fractions, which contained less than 1% of the radioactivity, were discarded. After adjusting the pH to 3 with 1 N hydrochloric acid, the homogenates were extracted five times with water-saturated butanol. Ninety-five per cent or more of the radioactivity was recovered in the butanol phase. The pooled butanol extracts were back extracted with water and evaporated to dryness in a stream of air at room temperature. The residue was dissolved in butanol saturated with concentrated ammonium hydroxide and chromatographed, as previously described, except that dioxane was omitted from the butanol-ammonia developer (Albright, Larson and Deiss, 1953). After the chromatograms were developed they were cut into 3 mm. sections (usually 45–50 per chromatogram) and counted in an automatic sample changing end window Geiger counter. Each section was counted for a total of 320 counts to obtain satisfactory statistical reliability. (Background average: 12 counts per minute.) After counting the sections were reassembled and the position of the carrier thyroxine and triiodothyronine determined by spraying with diazotized sulfanilic acid. Coincidence of the radioactivity with the carrier triiodothyronine spot was taken as an indication of deiodination of labeled thyroxine to triiodothyronine.[7]

In calculation of the quantity of triiodothyronine derived from the labeled thyroxine it was assumed that the thyroxine was randomly labeled at the 3′ and 5′ positions. It follows then that only one half of the triiodothyronine derived from thyroxine would be labeled. The quantity of labeled thyroxine originally present would be the sum of the thyroxine activity remaining plus twice the activity present as triiodothyronine. Hence the percentage of triiodothyronine derived from thyroxine can be expressed as follows:

$$\% \text{ triiodothyronine derived from thyroxine} = \frac{2 \times \text{triiodothyronine radioactivity}}{2 \times \text{triiodothyronine radioactivity} + \text{thyroxine activity}} \times 100.$$

[4] Obtained from Abbott Laboratories, Oak Ridge, Tennesee.

[5] Obtained through the courtesy of Dr. M. J. Schiffrin, Hoffmann-LaRoche, Incorporated.

[6] Obtained through the courtesy of Mr. Weyland Sears, E. R. Squibb and Sons.

[7] Identity of the labeled triiodothyronine was confirmed by column chromatography by Dr. John F. Morrissey in our laboratory.

RESULTS

Representative experiments in the three groups of animals are illustrated in Figure 1. The hyperthyroidism in the experiment shown was cold induced, but the findings did not differ significantly from those in the animals fed desiccated thyroid. The hypothyroid animals were those which had been thyroidectomized. The striking difference in the deiodination of thyroxine to triiodothyronine in these three groups is evident, deiodination being greater than normal in the hyperthyroid and less than normal in the hypothyroid.

The appearance of labeled triiodothyronine with respect to duration of incubation in the three groups of animals is shown in Figure 2. Both the rate and extent of deiodination of thyroxine to triiodothyronine was greater in the hyperthyroid and less in the hypothyroid than in the normal. At the end of 12 hours of incubation the extent of deiodination of thyroxine to triiodothyronine was 50% in the cold induced hyperthyroidism, 30% in the normal and 5% in the thyroidectomized. At 9, 12 and 24 hours of incubation the animals treated with desiccated thyroid showed

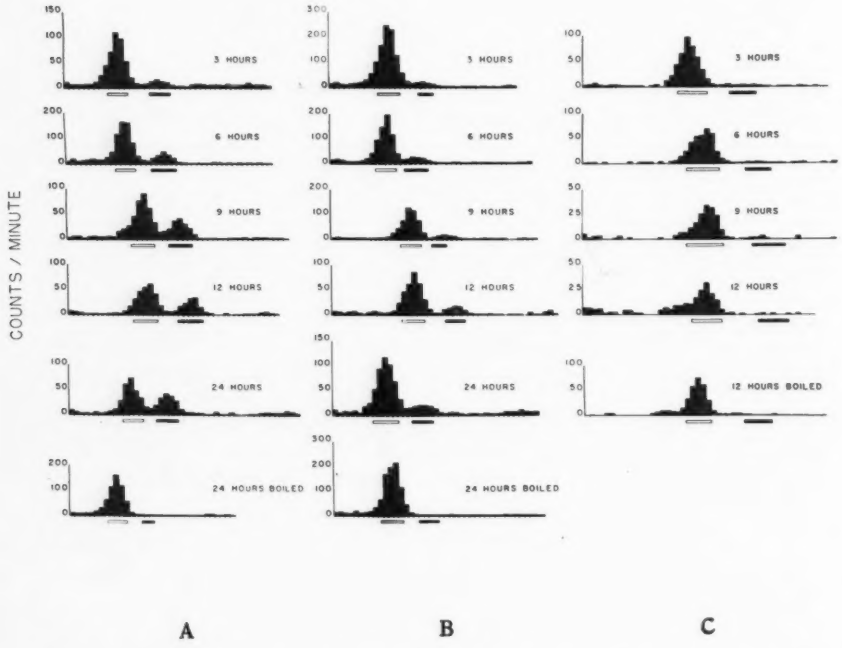

FIG. 1. Graph of chromatograms illustrating deiodination of thyroxine to triiodothyronine by rat kidney slices in (A) hyperthyroid, (B) normal, (C) thyroidectomized animals. Position of radiothyroxine is indicated by the open bar, that of radiotriiodothyronine by the solid bar.

somewhat less diodination than the animals exposed to cold, although more than the normals.

In the animals made hypothyroid by feeding thiouracil for 4 weeks the extent of deiodination of thyroxine to triiodothyronine was comparable to that seen in the thyroidectomized group, being 2% at the end of 12 hours of incubation. However, in the control experiment in which the animals were fed thioracil for only four days, similar results were obtained, suggesting the possibility of a direct inhibitory effect of the thiouracil on the thyroxine deiodinating system. This possibility was examined by the

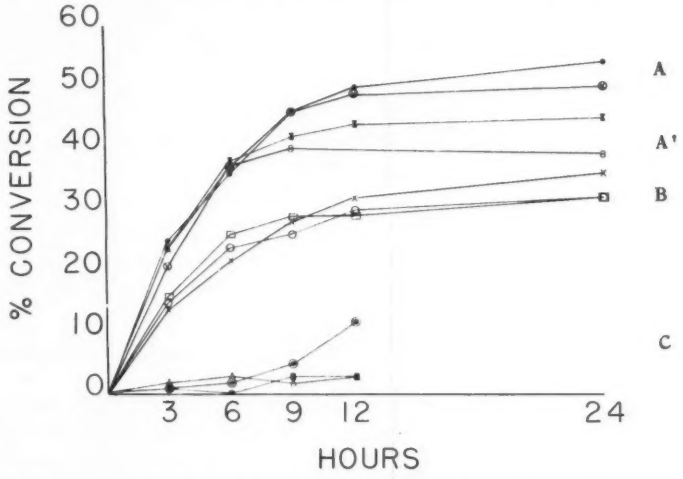

FIG. 2. Conversion of thyroxine to triiodothyronine by rat kidney slices related to duration of incubation in (A) cold induced hyperthyroid, (A') desiccated thyroid fed hyperthyroid, (B) normal, (C) thyroidectomized animals. Each curve represents an experiment using two rats.

addition of thiouracil in 3.1×10^{-6} and 3.1×10^{-7} molar concentration to the media containing normal kidney slices. As shown in Figure 3, a significant inhibition of thyroxine deiodination was observed under these conditions.

The addition of triiodothyronine in quantities of 0.01, 0.1 and 1.0 μg. directly to the reaction media reduced the thyroxine deiodination by normal kidney slices. In the experiments in which radioiodide was substituted for radiothyroxine no labeled thyronine compounds were identified.

DISCUSSION

Following the identification of triiodothyronine in human plasma by Gross and Pitt-Rivers (1951, 1952 a), several reports appeared which indicated that this compound has several times the metabolic activity of thyroxine. This conclusion was based upon comparisons of these two com-

pounds in (a) preventing goiter in thiouracil treated rats (Gross and Pitt-Rivers, 1952 b, 1953) (b) prevention of the hyperplasia and enlargement of basophil cells and degranulation of acidophilic cells in the pituitary of thiouracil-treated rats and (c) the effect on the metabolic rate and serum cholesterol in myxedematous patients (Gross, Pitt-Rivers and Trotter, 1952; Lerman, 1953; Asper, Selenkow and Plamondon, 1953). Rawson *et al.* (1953), on the other hand, concluded from their observations that the significant difference between these two compounds was in the speed in which the metabolic effects occurred. Triiodothyronine exerted an almost

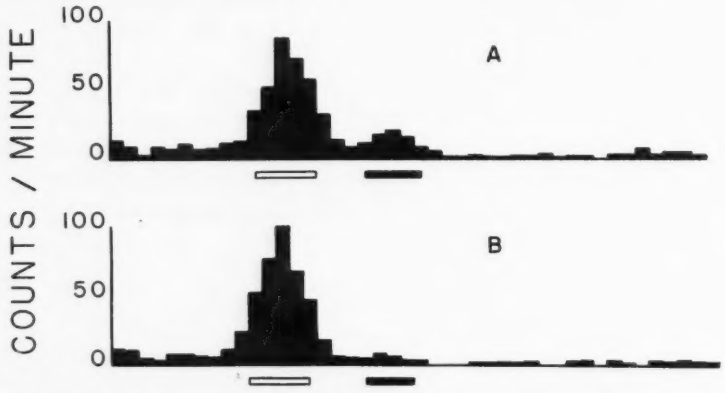

FIG. 3. Graph of chromatograms illustrating effect of thiouracil on thyroxine deiodination by kidney slices. (A)—indicates untreated normal control (B)—indicates addition of thiouracil in 3.1×10^{-7} molar concentration to reaction media containing normal kidney slices. Position of radiothyroxine is indicated by the open bar, that of radiotriiodothyronine by the solid bar.

immediate effect whereas the effects of thyroxine were slow in appearing and were more prolonged. Similar observations have been reported by Selenkow (1955) who found that the clinical effects of triiodothyronine could be observed within 4–6 hours following administration. Lerman (1953) has reported that the basal metabolic rate following treatment with triiodothyronine returned to pretreatment levels in from 7–9 days following the cessation of triiodothyronine. This is in marked contrast to the slow fall in metabolic rate which follows the withholding of thyroxine.

Thyroxine, unlike triiodothyronine, is bound to a serum alpha globulin (Larson, Deiss and Albright, 1952; Deiss, Albright and Larson, 1953; Larson, Deiss and Albright, 1954) which probably alters its diffusibility. This may in part account for the observed differences in the speed of action. The possibility arises, however, that thyroxine per se has no metabolic activity except as a precursor of triiodothyronine, the latter being derived from thyroxine by slow deiodination in peripheral tissues. There are several observations which support this view. First, thyroxine is present in serum in greater quantities than triiodothyronine. Secondly, when la-

beled thyroxine is given to thyroidectomized mice (Gross and Pitt-Rivers, 1952 a) or propylthiouracil treated rats (Kalant, Lee and Sellers, 1955), labeled triiodothyronine can be recovered from the plasma and tissues. Thirdly, butyl-4-hydroxy-3:5-diiodobenzoate has been shown to antagonize the metabolic action of thyroxine (Sheehan, Wilkinson and MacLagan, 1951) and to enhance the action of triiodothyronine (MacLagan, Sprott and Wilkinson, 1952) which it does presumably by inhibiting, competitively, the deiodination process (Wilkinson, Sprott, Bowdon and MacLagan, 1954). Finally, *in vitro* deiodination of thyroxine by kidney slices has been demonstrated (Albright, Larson and Tust, 1954).

In an earlier report (Albright, Larson and Tust, 1954) deiodination of thyroxine was observed in normal rat kidney slices. The reaction proceeded slowly and continued for as long as 12 hours. The system was heat labile and presumably enzymatic. No activity could be demonstrated after five minutes of boiling. The system therefore differs from that described by MacLagan (1954) which requires two hours of boiling for inactivation.

In the present experiments the deiodination system had all of the characteristics observed in the preliminary studies. Furthermore, triiodothyronine added to the media was found to have an inhibiting effect on thyroxine deiodination. Radioiodide substituted for radiothyroxine resulted in no labeling of triiodothyronine. These observations provide evidence that the appearance of labeled triiodothyronine in the experimental animals is not the result of a simple exchange reaction.

Extension of the observations to animals with different thyroid gland activities has disclosed a direct relationship between the level of thyroid activity and the capacity of kidney slices to deiodinate thyroxine. The tissues of the hyperthyroid animals, whether the state of hyperthyroidism was induced by thyroid feeding or exposure to cold, had more active deiodinating systems than the normal animal. The kidney slices of athyreotic animals, on the other hand, were found to have almost no deiodinating activity. In view of these findings it is postulated that an adaptive enzyme exists which is conditioned by the level of thyroxine presented to the tissue.

The inhibitory effect of thiouracil on the deiodination of thyroxine is of interest. It suggests the possibility of a peripheral site of action of this group of anti-thyroid compounds. If this were important clinically, however, a more immediate response to thiouracil treatment would be expected.

The significance of the presence of the deiodinating system in the kidney is not known. It is interesting that Van Arsdel *et al.* (1954) found both thyroxine and triiodothyronine to be present in high concentration in this organ. A number of other tissues have been examined *in vitro*. These included somatic muscle, cardiac muscle, diaphragm, liver, spleen, adrenal and salivary gland. Of these tissues only cardiac muscle showed significant deiodinating activity (Morrissey, Larson and Albright, 1955).

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SUMMARY AND CONCLUSIONS

Previously reported deiodination of thyroxine to triiodothyronine *in vitro* by rat kidney slices is confirmed. The deiodinating system is heat labile, and is inhibited by thiouracil and triiodothyronine.

The deiodination of thyroxine by kidney slices of rats with varying thyroid function was studied.

Kidney slices of hyperthyroid rats showed greater deiodinating activity and hypothyroid rats less than normal.

The presence of an adaptive thyroxine deiodinating enzyme system conditioned by the level of thyroxine in the tissue is postulated.

Acknowledgment

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MECHANISM OF BLOCKADE OF PITUITARY ACTIVATION IN THE RAT BY MORPHINE, ATROPINE AND BARBITURATES[1]

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PITUITARY activation, resulting in the release of ovulating hormone (LH or gonadotrophin), occurs in the female rat on the day of proestrus. Under controlled lighting conditions the activation process transpires between 2–4 P.M. and it may be interrupted by such seemingly unrelated agents as barbiturate anesthetics (Everett and Sawyer, 1950), anticholinergic and antiadrenergic agents (Everett *et al.*, 1949; Sawyer *et al.*, 1950, 1951) and the analgesic drug, morphine (Barraclough and Sawyer, 1955). Blocking nervous activity at presumably different sites, these effective agents have, by their multiplicity, made it difficult to analyze the process of neurogenic stimulation of the adenohypophysis. It should be emphasized, however, that a wide variety of other nerve-blocking agents are ineffective in preventing the release of ovulating hormone in the rat (Sawyer, *et al.* 1950, 1951), and that some of the effective agents have blocked the process in other "spontaneously" ovulating forms such as the hen (Zarrow and Bastian, 1953) and cow (Hansel and Trimburger, 1951).

Recent electroencephalographic (EEG) studies have indicated certain similarities in the effects of atropine, morphine and barbiturates on the EEG (Wescoe *et al.*, 1948; Wikler, 1950, 1952a, b; Funderburk and Case, 1951). Recording from the cerebral cortex in dogs and cats, these investigators have detected a decrease in frequency and an increase in amplitude and synchrony of the electrical waves following administration of these agents. In unrelated work, other investigators have demonstrated that anesthetics elevate the threshold of EEG arousal of the reticular formation of the brain stem (French *et al.*, 1953; Arduini and Arduini, 1954; King, 1954). The present work applies the techniques of EEG recording from both the superficial cerebral cortex and the deeper preoptic and hypothalamic regions of the rat brain stem, as well as methods of electrically

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stimulating the reticular formation, in an effort to determine the sites and mechanisms of action of the agents effective in blocking the release of ovulating hormone from the pituitary.

The results reveal that during a period in which a particular dose of atropine, morphine or nembutal is effective in blocking pituitary activation it slows and amplifies the waves not only in the electroencephalogram (EEG) but also in the electrohypothalamogram (EHG). These slow waves do not occur, however, following injection of the adrenergic blocking agent SKF-501 (N- [9-fluorenyl]-N-ethyl-β-chlorethylamine). Effective doses of atropine, morphine and nembutal, but not SKF-501, elevate the threshold to electrical arousal of the reticular formation. It would appear that the first three agents may have a common central site or mechanism of action, while SKF-501 acts in a different manner or at a different site or both.

MATERIALS AND METHODS

Sprague-Dawley female rats ranging in weight from 200–300 gms. and maintained under the same conditions as those in the preceding paper (Barraclough and Sawyer, 1955) were employed in the present study.

Under ether anesthesia the rat was fixed in a stereotaxic device and the skull trephined with a dental drill. The effects of ether were largely dissipated before recording began, and the cut skin surfaces were treated with local procaine. Electrical recordings were made with an 8-channel Grass amplifier and ink-writer. EEG arousal was induced by electrical stimulation of the central cephalic brain stem at a frequency of 300/sec. and a shock duration of 0.5 ms. with a Grass stimulator. In the latter experiments curare was employed to avoid motor artifacts, and respiration was maintained via tracheotomy tube with a respiration pump. The usual dose of *d*-tubo-curarine was 0.3 mg. intravenously and 0.6 mg. intraperitoneally supplemented from time to time with another 0.6 mg. intraperitoneally.

Bipolar concentric needle electrodes were used to record from and to stimulate the brain stem, and unipolar phonograph needle electrodes served to record from the cerebral cortex. Deep electrode sites were confirmed by a histological study of the fixed brains.

The drugs employed include nembutal (sodium pentobarbital), phenobarbital, morphine sulfate, atropine sulfate and SKF-501 (N-[9-fluorenyl]-N-ethyl-β-chlorethylamine). Except for the last, which was injected intravenously, they were all administered subcutaneously in doses to be reported below.

RESULTS

Barbiturates. The effects of nembutal were observed in several rats. Within a few minutes after the injection of a light anesthetic dose known to block pituitary activation (30 mg./kg.), the electrical waves in both the cerebral cortex and brain stem are decreased in frequency and increased in amplitude, and the heart is slowed. As illustrated in Figure 1, the effect lasts at least two hours. During this period strong afferent stimulation such as pinching the tail fails to arouse the EEG. Examples of positive arousal records will be illustrated in later sections. The well-

known phenomenon of barbiturate elevation of the threshold of electrical arousal in response to stimulation of the reticular formation was confirmed in two rats. An hour after nembutal injection the threshold of arousal on stimulating the midbrain reticular formation, was raised about 100% from approximately 0.5 volt to one volt.

Phenobarbital, at a dosage also known to block pituitary activation, 50 mg./kg. (Everett and Sawyer, 1950), was observed in two rats to exert an effect on the EEG and EHG records similar to that of nembutal. Strong

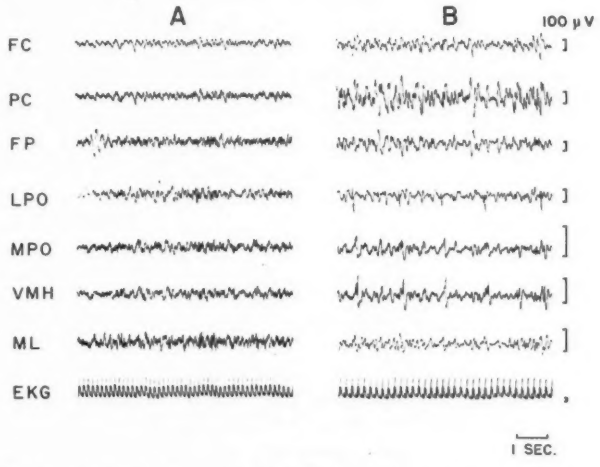

FIG. 1. Effects of nembutal on the electrical activity of the rat cerebral cortex and brain stem. A. Before nembutal. B. Two hours after nembutal, 30 mg./kg. subcutaneously. Abbreviations in this and subsequent figures: EKG, electrocardiogram. DHA, dorsal hypothalamic area. DMH, dorsomedial hypothalamic nucleus. FC, frontal cortex. FP, frontoparietal cortical leads. LHA, lateral hypothalamic area. LPO, lateral preoptic area. ML, lateral mammillary area. MM, medial mammillary area. MPO, medial preoptic area. PC, parietal cortex. SMA, supramammillary area. SO, supraoptic hypothalamic nucleus. VMH, ventromedial hypothalamic nucleus. μV, microvolts.

afferent stimulation failed to arouse the EEG but electrical stimulation of the reticular formation was not attempted in these experiments.

Morphine. Records were made of the effects of various dosages of morphine in 10 rats. It will be remembered that 20 mg./kg. blocks pituitary activation if injected at the onset of the sensitive period on the day of proestrus (Barraclough and Sawyer, 1955), while 50 mg./kg. maintains a blocking level over many hours.

Figure 2 illustrates the effects of 20 mg./kg. morphine on the electrical record of cortex and brain stem. Within a few minutes after injection the waves in the parietal cortex were slowed in frequency and increased in amplitude. In half an hour similar changes were observed in the preoptic and hypothalamic channels. The alterations were maintained for more than

90 minutes (Fig. 2B). However, by 160 minutes (Fig. 2C) at which time the pituitary blocking effect of 20 mg./kg. had dissipated, the electrical activity in channels 4–7 had returned to preinjection fast low-amplitude characteristics. The parietal cortex still showed the effects of morphine at this time. To test the similarities of the effects of morphine and nembutal in the same individual, 50 mg. nembutal was injected intraperitoneally two minutes after the record in Figure 2C. Three minutes later (Fig. 2D). the slow high-amplitude activity had returned to channels 4–7, the record

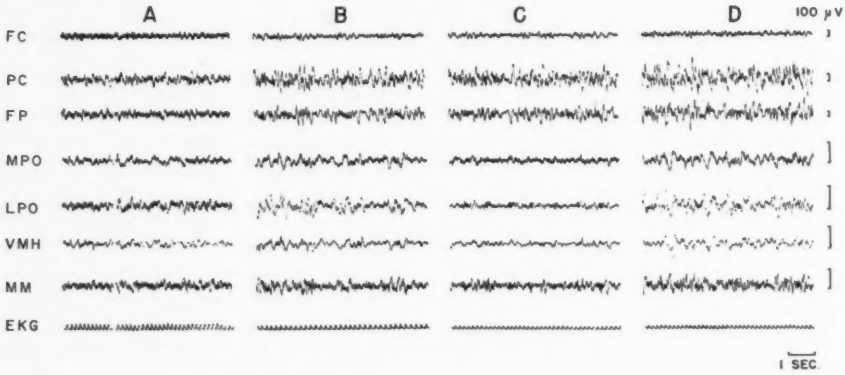

FIG. 2. Effects of morphine sulfate (20 mg./kg., subcutaneously) on the electrical activity of the rat cerebral cortex and brain stem. A. Before morphine. B. 90 minutes after morphine. C. 160 minutes after morphine. D. 5 minutes after C and 3 minutes after nembutal, 50 mg./kg., intraperitoneally.

resembling Figure 2B very closely. Neither morphine nor nembutal appeared to affect the activity in the frontal cortex (channel 1) in this experiment.

Weaker, non-blocking dosages (as far as release of pituitary gonadotrophin is concerned), e.g. 10–12 mg./kg., were observed to affect the electrocortical record in a manner similar to the changes in channel 2 of Figure 2, but to exert practically no influence on preoptic or hypothalamic activity. In these preparations the latter areas were affected in the characteristic way by additional injections which brought the total dosage above 20 mg./kg.

Higher dosages (50 or 100 mg./kg.) slowed the waves in all channels within a few minutes. The tendency could be temporarily reversed and the animal aroused by applying such strong afferent stimulation as pulling the tail. After about an hour, intermittent bursts of spikes were superimposed upon or interspersed with the slow waves. These bursts gradually became more frequent in all channels (e.g. ventromedial hypothalamic nucleus, Figure 3)and during the interval from 2–4 hours after injection they coalesced repeatedly into brief periods of generalized seizure activity.

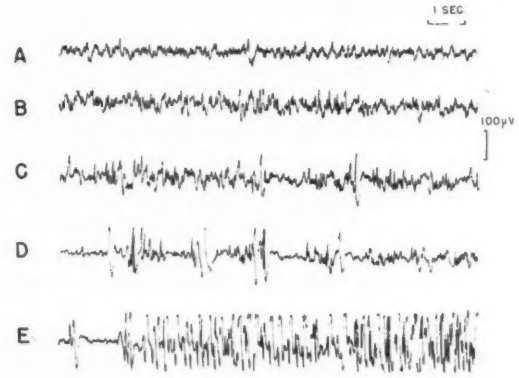

FIG. 3. Effects of morphine sulfate (100 mg./kg., subcutaneously) on the electrical activity of the ventromedial hypothalamic nucleus. A. Before morphine. B. 45 minutes after morphine. C. 100 minutes after morphine. D. 180 minutes after morphine. E. 200 minutes after morphine.

The motor manifestations of these electrical seizures were slow writhing movements and marked tail extension, perhaps the equivalent in the rat of the Straub-tail reaction of the mouse (Krueger *et al.*, 1941).

These effects of morphine on the rat EEG confirm the findings of Cahen and Wikler (1944) and extend them by adding information from electrode placement deep in the brain stem.

Figure 4 illustrates the effects of 25 mg./kg. morphine in a curarized

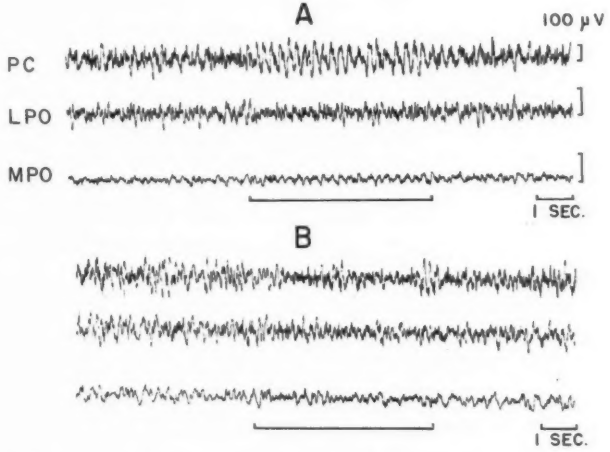

FIG. 4. Effects of morphine sulfate (25 mg./kg., subcutaneously) on the electrical activity of the rat brain and on the arousal reaction to stimulation of the midbrain reticular formation. Stimulation during the underlined periods, 0.5 volt. A. Before morphine. B. 90 minutes after morphine.

rat not only upon the resting electrical activity but also upon the threshold of arousal by electrical stimulation of the midbrain reticular formation. Characteristic of further arousal in the already reasonably alert parietal cortex and medial preoptic region (Fig. 4A) is the appearance of a theta type (5–7/sec.) rhythm; in the lateral preoptic area arousal reduces the amplitude and increases the frequency of the waves. Ninety minutes after this dose of morphine the same stimulus (0.5 volt) not only fails to evoke the theta rhythm, but it scarcely brings the electrical record to the degree of alertness of the resting control condition in Figure 4A. At this stage a stimulus approximately 50% stronger was required to evoke the same

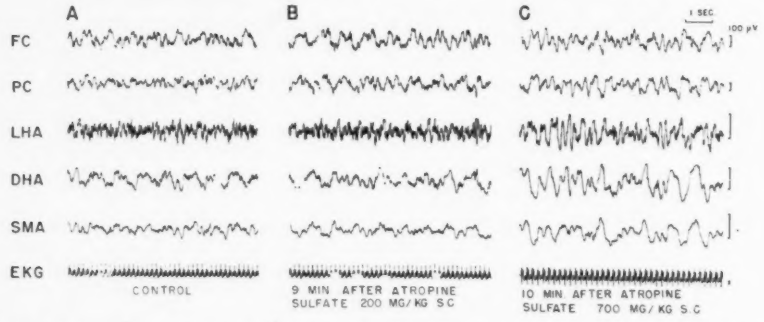

FIG. 5. Effects of atropine sulfate on the electrical activity of the rat brain. Cumulative dosages are given on the figure. C. 20 minutes after B, with additional 500 mg./kg atropine in meantime.

electrical response as that observed prior to morphine injection, e.g. from 0.4 volt before to 0.6 volt after morphine. A similar degree of elevation of threshold has been observed in rabbits (Sawyer, 1955b).

Atropine. Rats tolerate relatively tremendous dosages of atropine and the usual dose employed to block release of pituitary gonadotrophin has been 700 mg./kg. s.c. The threshold level for blocking this phenomenon is about 230 mg./kg. (Everett and Sawyer, 1953). The effects of various amounts of atropine on the electrical activity of the brain were observed in 8 rats.

Figure 5 illustrates the effects on electrical activity of a non-blocking dose (200 mg./kg.) and a blocking level (700 mg./kg.). It is apparent that after the latter, but not the former, there is a marked slowing and increase in amplitude in all channels. These effects have been evoked by as little as 250 mg./kg. s.c. after about half an hour. Even with the larger dosage given subcutaneously the high amplitude slow waves do not develop for some minutes. They are produced within a matter of seconds, however, by giving one-tenth of the subcutaneous dose (70 mg./kg.) intravenously. In three of the rats with the higher subcutaneous dosage bursts of spikes coalesced into generalized seizures similar to those encountered with

higher dosages of morphine. Their motor symptoms also resembled those in morphine-treated rats in that they displayed the tail extension phenomenon.

Higher dosages of atropine also raise the threshold of arousal. Sensory stimuli such as clapping, pinching the tail or inserting a rectal thermometer, which before atropine induced electrical arousal lasting for as long as a half minute, either failed completely to arouse or were effective only very briefly after 700 mg./kg. The threshold of arousal by electrical stimulation of the midbrain reticular formation was determined in two curarized rats before and 10 minutes after 350 mg./kg. atropine (35 mg./kg. i.v. and 315 mg./kg. s.c.). As in the case of morphine the threshold was raised about 50%. This atropine-induced elevation of threshold of arousal by electrical stimulation of the midbrain reticular formation has also been confirmed in 3 rabbits (Sawyer, 1955b).

SKF-501. The standard dose of this potent adrenergic blocking agent employed in preventing pituitary stimulation has been 10 mg./kg. i.v. (Sawyer *et al.*, 1950). A dose of 5 mg./kg. is partially effective in this capacity (Sawyer and Barraclough, unpublished). The effects of 5–10 mg./kg. i.v. SKF-501 on the electrical record have been tested in several rats.

In the non-curarized rat even the higher dosage fails to induce any noticeable changes in electrical activity of the cortex or brain stem, either initially or after 90 minutes, by which time its greatest antiadrenergic capacity is reached (Nickerson, 1949). In the curarized animal SKF-501 at 10 mg./kg. i.v. rapidly produces in all channels a generalized depression in amplitude characteristic of a marked lowering in blood pressure. The effect is quite unlike those exerted by nembutal, morphine or atropine in that the frequency was unchanged while the amplitude was greatly reduced.

In the curarized rat 5 mg./kg. i.v. SKF-501 fails to affect the electrical activity of the brain at rest (Fig. 6). The threshold of electrical arousal remained the same before and one hour after SKF-501 (<1 volt) and Figure 6 shows the parallel dramatic response to stimulating the midbrain reticular formation for 5 seconds with 2.2 volts. The marked theta rhythm in all channels continued for about 15 seconds after termination of stimulus in each case. In the rabbit which will remain quiescent without curare, 10 mg./kg. i.v. fails to change the electrical record or the threshold of arousal (Sawyer, 1955b).

DISCUSSION

Drugs which affect nerve function generally act at numerous sites or levels in the central or the peripheral nervous system. Certain dosages of atropine, morphine and the barbiturates have well-known differential actions at various loci. The present study reveals that dosages of these three unrelated agents which block the release of pituitary gonadotrophin also

depress the reticular activating system (Magoun, 1952), to the extent that a sleep-like electrohypothalamographic record is produced and upon direct electrical stimulation of the midbrain reticular formation the threshold of arousal is considerably elevated. Weaker dosages fail to evoke one or more of these three correlated phenomena. It seems logical to assume that the relationship may be causal: that blockade of pituitary activation by the drugs may be related to their demonstrated effect on the reticular activating system.

Some of the implications of this assumption are as follows: (1) An "active" reticular activating system is necessary for a central timing mechanism or afferent stimulation to excite hypothalamic centers con-

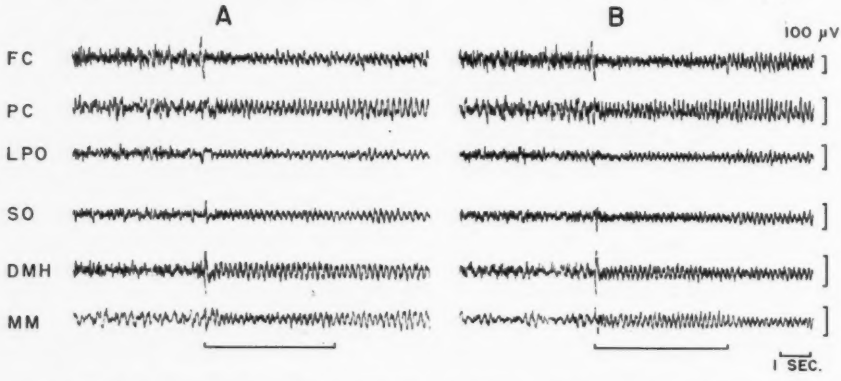

FIG. 6. Failure of SKF-501 to affect the electrical activity of the rat brain or the arousal response to stimulation of the midbrain reticular formation. Stimulation during the underlined periods, 2.2 volts. A. Before SKF-501. B. 60 minutes after SKF-501, 5 mg./kg., intravenous.

trolling adenohypophyseal function. (2) The role played by the reticular system in this sequence may not be a specifically excitatory one but perhaps "permissive" in the same sense as the term is applied in endocrine secretion (Ingle, 1954). (3) Nevertheless, an important function in the control of neuroendocrine secretion would be added to the weighty responsibilities of the reticular activating system. (4) Other drugs which raise the threshold of arousal may be found to block pituitary activation.

In view of evidence that the theta-type rhythm, which is so marked in the aroused rat EEG (Fig. 6), emanates from the hippocampus (Green and Arduini, 1954), one must consider the possibility that arousal of this rhinencephalic area is essential for pituitary activation. Another rhinencephalic structure whose almost differential activation leads to release of pituitary ovulating hormone in the rabbit is the olfactory bulb (Sawyer, 1955a). "Intrinsic olfactory activity" is apparently transmitted to the hypothalamus via the medial forebrain bundle. Electrical activity thought

to be functional in evoking the release of pituitary gonadotrophin in the cat has recently been recorded from the latter area on vaginal stimulation (Porter, Cavanaugh and Sawyer, 1954). The relative importance of these rhinencephalic structures and projections and the midbrain reticular formation to pituitary activation must be determined in later work.

It seems probable that the mechanism which prevents neurogenic stimulation of the release of pituitary gonadotrophin may also block ACTH release. Briggs and Munson (1954a, b) have recently reported that morphine, in doses shown above to affect the reticular system inhibits stress-induced release of pituitary ACTH. Nembutal also is known to block the effects of some non-specific stressing agents or situations. Morphine and nembutal as well as atropine may act themselves as stressing agents. The fact that morphine and nembutal also block the stress response weakens still further the argument of Dordoni and Timiras (1952), already answered by Everett (1952), that stressing agents block gonadotrophin release merely by stimulating ACTH secretion. How then would blockade of ACTH release be explained?

To say that the action of atropine is central rather than peripheral does not necessarily deny that its underlying mechanism may depend on its anticholinergic function, an attribute emphasized earlier (Sawyer *et al.*, 1949). Indeed, similar anticholinergic actions have been described for nembutal (Emmelin, 1941). On the other hand morphine's anticholinesterase activity (Eadie, 1941) would place it on the other end of the spectrum, as a drug with cholinergic tendencies.

The failure of SKF-501 to change the EEG record or the arousal threshold implies that its blocking action is exerted at a later stage in the pituitary activating mechanism. Further evidence will be presented in another publication (Sawyer, 1955b) that adrenergic blockade is exerted at the level of the pituitary itself.

SUMMARY

Dosages of nembutal, morphine or atropine which block neurogenic stimulation of the release of pituitary ovulating hormone in the rat have the following actions in common: (1) They induce high amplitude slow waves in the electrical record of activity in both brain stem and cerebral cortex. (2) They raise the threshold of EEG arousal in cortex and brain stem on direct stimulation of the midbrain reticular formation. Dosages of any of the three drugs which were originally too weak to cause the electrical changes or whose slowing effects on the electrical record wore off before the period of neurogenic stimulation, failed to block the ovulatory mechanism. The results strongly suggest that blockade of pituitary activation by these drugs is related to their demonstrated common effects on the central nervous system.

The antiadrenergic agent, SKF-501, blocks pituitary activation without

changing the electrical record or threshold of arousal on stimulating the reticular formation. Compared with the other drugs it may act in a different maner or at a different site or both, perhaps via adrenergic blockade at the adenohypophysis itself.

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THE EFFECT OF HYPOTHALAMIC LESIONS ON THYROID FUNCTION IN THE DOG[1,2]

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IT HAS been suspected for some time that the thyroid was at least in part under the control of the nervous system (Uotila, 1940). However, it remained for Greer (1952) to show that anterior hypothalamic lesions in rats prevent the usual thyroid hypertrophy upon the administration of propylthiouracil. Because his animals also showed normal thyroid-serum iodide ratios, he postulated the existence of two thyrotrophic hormones. Bogdanove and Halmi (1953) have since confirmed these results, but feel that they can be interpreted as due only to a decrease in the amount of thyrotrophin released in response to propylthiouracil administration. No studies are at present available on the effect of hypothalamic lesions on the thyroid in animals that have not been treated with propylthiouracil. In the course of other investigations on the relationship of the hypothalamus to the anterior lobe of the pituitary, the following studies were carried out in dogs. It was felt that they were of interest because they show the effect of hypothalamic destruction on thyroid function in the absence of any modifying agents.

MATERIALS AND METHODS

Adult mongrel dogs of both sexes, weighing 10-14 kg., were used in this study. They were maintained throughout on a diet of "Ken-L-Biskit,"[6] each dog receiving 200 gm. of this ration per day. This dietary regimen gave the animals approximately 60 μg. per day of iodine intake. The dogs remained in good condition throughout the study.

Radioactive iodine uptakes were determined by a technique described elsewhere (Fredrickson, Ganong, and Hume, 1955). All animals received 3–5 μc. of radioactive iodine intravenously. In most cases thyroid uptake of iodine was determined at 24, 48, and 72 hours after injection. In a few animals, uptakes were determined at 24, and 48 or only at 24 hours.

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The 23 dogs which are the subject of the present report had hypothalamic lesion produced by a technique which will be described in detail elsewhere. Using a stereotaxic machine, a radiopaque dye was injected into the third ventricle, needles were inserted into the hypothalamus under x-ray control and lesions were then produced by electrolysis. The animals were allowed to recover for a period of three weeks, and at the end of this time, the uptake of radioactive iodine was determined. After the uptakes were completed, the dogs were tested for their eosinopenic response to immobilization and operative trauma ("standard operation," Hume, 1953). One month after the production of their lesions, they were subjected to left adrenalectomy. They then received either no treatment or intramuscular hydrocortisone acetate for two weeks, at which time their right adrenals were surgically removed and the animals were killed. Six dogs received 50 mg. of hydrocortisone daily, and three 6.25 mg. every other day for the two week period. Unpublished data from this laboratory (Ganong, 1954) show that hydrocortisone in the dosages used has no effect on thyroid or gonadal weight and histology. The studies of compensatory adrenal hypertrophy and atrophy have been the subject of a previous report (Ganong and Hume, 1954).

At the time of autopsy, the animals were anesthetized with nembutal and perfused through the carotid arteries with saline followed by 10% neutral formalin to fix the brain and pituitary in situ. Complete post-mortem examinations were then performed. The thyroid and gonads were dissected clean of fat and connective tissue, weighed and fixed in Zenker's solution for histologic examination. The hypothalamus and pituitary region were removed "en bloc" and fixed in formalin for serial sectioning. Thyroids and gonads, as well as the pituitary-hypothalamic block, were stained with hematoxylin and eosin. Drawings were made of the lesion as located by the serial sections of the diencephalon, and the lesions in each animal were then plotted on a composite diagram of a sagittal section of the dog hypothalamus. In this manner, it was possible to reconstruct all the lesions and to determine the areas common to them.

RESULTS

The mean uptake of radio-iodine by the thyroid in normal dogs was 18.7, 27.2 and 31.5% of the injected dose at 24, 48 and 72 hours after injection. Although there was moderate variation in the normal uptake, the lowest values seen in 40 normal animals were 2.8, 6.6, and 6.8% at 24, 48, and 72 hours respectively. Uptake values in hypophysectomized dogs fall well below these levels (Fredrickson, *et al.*, 1955). It is apparent from the data in Figure 1 that while the uptake of most of the dogs with hypothalamic lesions fell well within the normal range, there was definite depression of the thyroid uptake in a number of animals. Changes in thyroid histology correlated with this depression. In all animals in which the uptake was depressed, the thyroid showed definite atrophy (Fig. 2), while in the remainder it was normal. The mean thyroid weight in the dogs with depressed uptakes of I^{131} was 63.4 ± 3.0 mg./kg. (standard error of the mean), compared to 85.7 ± 6.3 mg./kg. in the animals with lesions which had normal I^{131} uptakes and 84.4 ± 5.4 mg./kg. in 18 normal dogs. The difference between these weights is not statistically significant, however, because of the large variability in thyroid weights found in normal dogs.

A few of the animals had their thyroid uptake of I^{131} measured before and after the production of their lesions, although this was not done routinely.

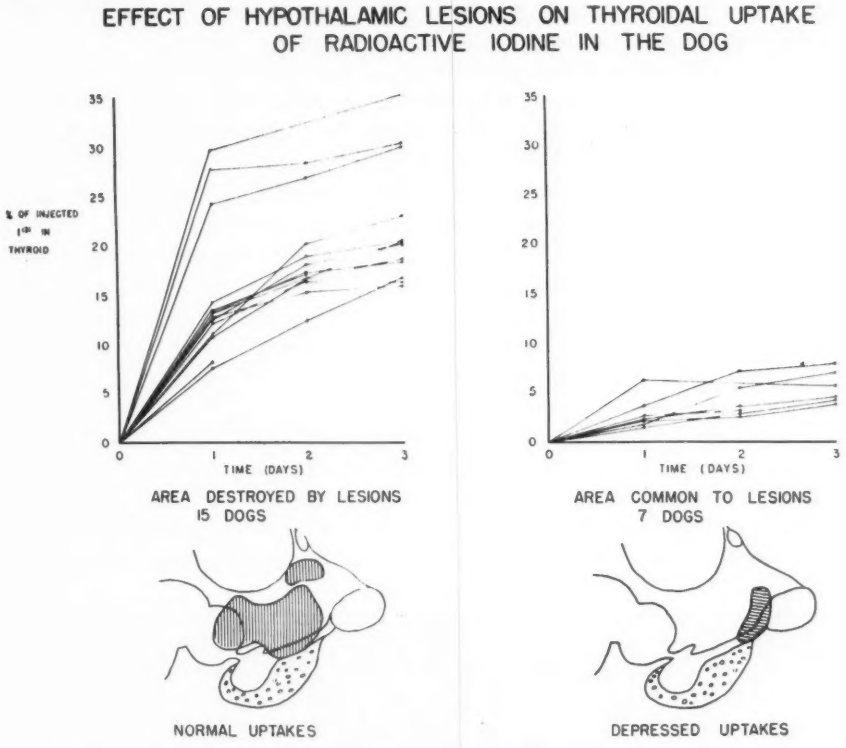

FIG. 1. Location of hypothalamic destruction in dogs with normal and low iodine uptakes.

Two typical animals are shown in Fig. 3. In Dog 58, there was no significant difference between the two uptakes. On the other hand, in Dog 40, there was a marked depression of the uptake after the lesion.

In order to compare the site of the hypothalamic destruction in these animals, they were divided into two groups, those with uptakes of less than 7% at 72 hours, and those with uptakes greater than 15%. Two dogs with uptakes of 9.6 and 7.8% at 72 hours were omitted from this part of the study, although they were included in the rest of the analysis. The dogs with uptakes of less than 7% all had lesions in an area located at the anterior end of the median eminence and extending somewhat upward from there. On the other hand, the dogs with normal thyroid function had lesions involving the middle and posterior portion of the median eminence, the mamillary bodies, and the region just below the anterior commissure. In some instances, lesions which encroached on the anterior part of the median eminence did not block the iodine uptake, but in general, in the animals with normal uptakes, there was strikingly little involvement of the area

which was involved in those dogs in which the thyroid function was depressed. Location of this area on the sagittal projection of the dog hypothalamus is illustrated in Figure 1.

It should be emphasized that thyroid function was not evaluated in these dogs until at least three weeks after the production of their lesions. This interval is important because it permits edema about the lesion to subside and temporary disruption of function beyond the actual area of destruction to regress. Failure to take this factor into account may explain some of the discrepancies in localization of areas involved in pituitary control (Greer, 1952; deGroot and Harris, 1952; McCann, 1952). The time interval also rules out the possibility of acute pituitary damage without histologic change which could be present when animals with hypothalamic destruction are tested for endocrine responses shortly after the production of their lesions.

In all dogs, the state of thyroid function was correlated also with the state of gonadal and adrenal function, as measured by gonadal weight and histology on the one hand and adrenal histology and weight as well as the eosinopenic response to stress on the other. From such an analysis, it is apparent that there was no consistent correlation between the status of the

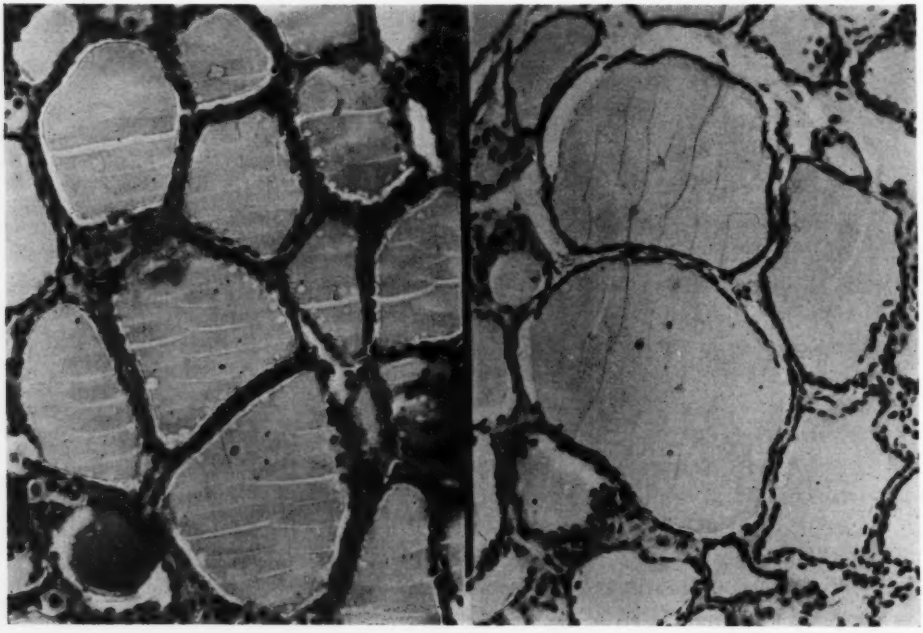

FIG. 2. Photomicrographs of the thyroid in dogs with hypothalamic lesions. The thyroid on the left was associated with a normal and the one on the right with a depressed iodine uptake. H and E stain. ×580.

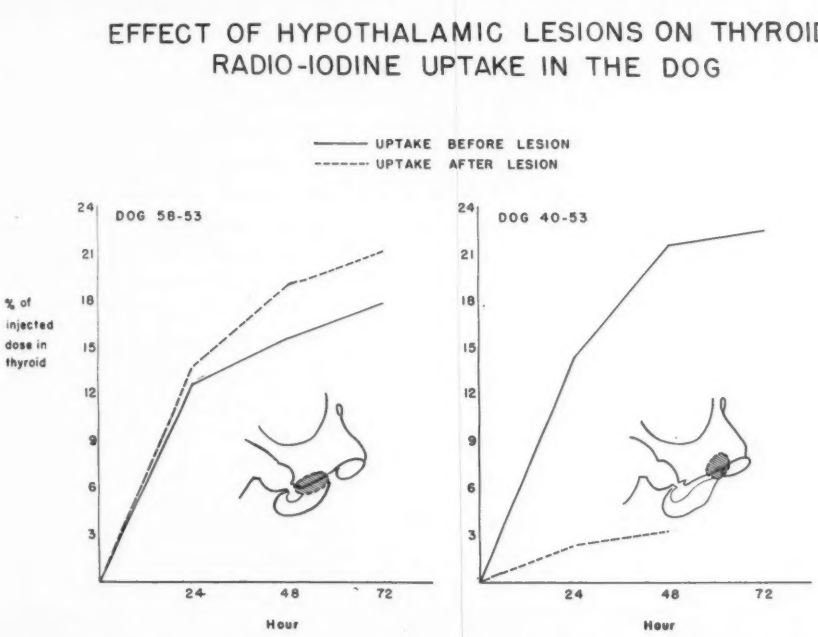

FIG. 3. Radioactive iodine uptake before and after the production of hypothalamic lesions. The shaded areas at the bottom outline the sites of hypothalamic destruction.

thyroid and the status of the other pituitary end organs. Thyroid depression usually occurred with gonadal or adrenal depression, but loss of stress responses, gonadal depression, and in one animal, thyroid depression did occur alone. The various combinations of end organ defects are summarized in Table 1.

TABLE 1. CORRELATION BETWEEN THYROIDAL, ADRENOCORTICAL AND GONADAL DEPRESSION IN 23 DOGS WITH HYPOTHALAMIC LESIONS

I. Iodine Uptake 8% at 72 hours

	Stress responses	
	Present	Absent
Gonads Normal	1	2
Gonads Atrophic	2	2

II. Iodine Uptake 8% at 72 hours

	Stress responses	
	Present	Absent
Gonads Normal	8	4
Gonads Atrophic	3	1

DISCUSSION

It is true that hypothalamic lesions which affect pituitary function in the dog are located in the median eminence and of necessity damage at least part of the portal hypophysial vasculature. It is possible, therefore, that the present results can be explained by pituitary damage secondary to encroachment on this source of pituitary blood supply. An experimental approach to rule out this factor is difficult, and at present, there are no conclusive data that pituitary dysfunction alone cannot explain the observed results. However, for a number of reasons, this seems an unlikely explanation.

In the first place, in all animals with depressed radioactive iodine uptakes, there were at least some portal hypophysial vessels remaining intact. Furthermore, not only did all animals have large amounts of grossly and histologically normal pituitary tissue present, but the small amount of pituitary damage sometives present bore no relation to the presence or absence of end organ deficits. Pituitary cell counts have not been done but it can be stated that eosinophiles and basophiles are both present in what appear to be roughly normal numbers. Another point of considerable interest is the fact that none of these animals showed adrenocortical atrophy histologically, and in only one of them was the adrenal weight less than that of the normal, unstressed dog. This observation has previously been reported in another series of animals from this laboratory (Ganong and Hume, 1954).

Section of the portal hypophysial blood vessels without damage either to the median eminence or the pituitary would provide an excellent experimental approach to this problem. Unfortunately, however, it is impossible to draw conclusions from the rather confusing literature (Uotila, 1940; Harris, 1948) because of a number of factors. In the first place, Harris and Jacobsohn (1952) have pointed out that the portal hypophysial vessels regrow with great ease. Therefore, in all studies in which their regrowth was not prevented by insertion of a large plate of some inert material, the results must be regarded as inconclusive. Furthermore, injection of the vessels with a material such as india ink, which permits their visualization, and serial section of the hypothalamo-pituitary region is essential if one is to conclude that the vessels are indeed completely interrupted. Many people have studied the problem of stalk section in the dog, but none have been able to perform the operation without considerable trauma to the pituitary area, and, in many instances, to the median eminence.

Similarly, the results of transplantation of the anterior pituitary to the eye or other locations remote from the diencephalon (Cheng, Sayers, Goodman, and Swinyard, 1949; McDermott, Fry, Brobeck, and Long, 1951; Harris and Jacobsohn, 1952) are difficult to interpret physiologically, because no matter how well vascularized these grafts may be, it has not

been proved that the observed results are not due to a decrease in the amount of functioning pituitary tissue.

Another approach to this problem is to study the animal in whom increasingly large amounts of pituitary tissue have been removed. From studies of this nature in the past (Aschner, 1912) it is now well accepted that one-third or less of the anterior lobe will support the thyroid, gonads and adrenals in the presence of a normal hypothalamus. On the other hand, the problem of what happens to the pituitary target glands with removal of more than two-thirds but less than all of the hypophysis has received little attention. Smith's classic work on the rat (1932) showed that instead of uniform, graded depression of thyroid, gonads and adrenals, the gonads atrophied while the thyroid and adrenals remained histologically normal when nine-tenths of the pituitary was absent. Keller and his colleagues (Keller, 1948; Keller, Lawrence and Blair, 1945) have similarly found that in the dog, as increasingly large amounts of pituitary tissue are removed, gonadal depression is first to appear, and adrenocortical last, adrenocortical function being supported by very small remnants of pituitary tissue. This problem has also been studied in the human, and here, isolated end organ deficits have been reported (Paschkis and Cantarow, 1951; Myers, Maddock, Leach and Klein, 1952). Sheehan and Summers (1949) point out, however, that valid conclusions can be drawn only from autopsied cases, since only in this manner can concurrent hypothalamic damage be ruled out. When this is done, the usual sequence of endocrine atrophy in man with increasing pituitary failure is gonads, thyroids, and lastly adrenals (Sheehan and Summers, 1949; Peters, German, Mann and Welt, 1954).

A study of the effect of subtotal hypophysectomy in the dog is now in progress in this laboratory. Preliminary data, based on 16 dogs, indicate that gonadal function is depressed one month after removal of three-fourths of the anterior lobe. Depression of thyroidal function also occurs after removal of four-fifths of the pars distalis while some adrenocortical function persists unless the removal is complete. It would be reasonable, therefore, to expect gonadal depression alone, or gonadal and thyroidal, or gonadal, thyroidal and adrenal, in that sequence if results in the lesion animals were due to pituitary damage. The dogs in the present study do not follow this pattern. Four had isolated loss of normal adrenocortical stress responses with no other abnormality, while two had thyroidal and adrenocortical deficiency combined. Obviously, more work must be done on this point before definitive conclusions are possible. However, it is clear that the end organ deficits in the dogs with hypothalamic lesions do not correlate with the spectrum of hypopituitarism.

On the other hand, a rough correlation was noted between the part of the hypothalamus destroyed and the particular endocrine deficit seen. Anterior median eminence destruction produced thyroid atrophy, destruction of the anterior and middle median eminence loss of the eosinopenic response to

stress, and caudal median eminence lesions gonadal atrophy. These localizations are consistent enough to suggest further investigation.

SUMMARY

1. Thyroidal uptake of radioactive iodine and thyroid weight and histology have been analyzed in 23 dogs with hypothalamic lesions and correlated with the eosinopenic response to stress, adrenal weight and histology, and gonadal weight and histology.

2. Five dogs showed definite depression of thyroidal I^{131} uptake, and histologic evidence of thyroidal atrophy. In these animals, lesions were localized to the region in and just above the anterior end of the median eminence. Thyroid function and histology were normal in animals with lesions elsewhere, including the posterior and middle portion of the median eminence.

3. There was no consistent correlation between thyroid depression and depressions of other pituitary controlled endocrines in the dogs with hypothalamic lesions. Rather, the type of endocrine picture produced correlated roughly with the site of the hypothalamic destruction.

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HISTOCHEMISTRY OF NORADRENALINE IN THE ADRENAL MEDULLA OF RATS AND MICE

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INTRODUCTION

HISTOCHEMICAL methods have recently been described demonstrating two different kinds of chromaffin cells in the adrenal medulla: 1) after fixation in formalin, some medullary cells exhibit a strong fluorescence in ultraviolet light and the remaining cells fluoresce but feebly (Eränkö, 1951, 1952); 2) treatment with potassium iodate selectively stains some medullary cells brown and the others remain almost colorless (Hillarp and Hökfelt, 1953). Determinations of adrenaline and noradrenaline in microdissected pieces of adrenal medulla, carried out after separation of these catechol amines by paper chromatography (Eränkö, 1954), showed that the noradrenaline content of those medullary cells of the cat and the hamster which fluoresce after formalin fixation is far higher than that of the remaining cells (Eränkö, 1955a, b). Strongly suggestive evidence of similar relations between the fluorescent medullary cells and noradrenaline has been obtained in studies with some other mammals (Eränkö, 1955c). Similar suggestive observations support the view that the medullary cells which give a positive iodate reaction also contain much noradrenaline (Hillarp and Hökfelt, 1953, 1954), but direct proof concerning the validity of this reaction has not been presented. The validity of the iodate reaction is nevertheless supported by the fact that both the fluorescence method and the iodate method have been seen to demonstrate selectively those medullary cells of the hamster's adrenal which are known to contain predominantly noradrenaline (Eränkö, 1955d). The present paper describes a study in which the two histochemical methods were applied to adrenals of the rat and the mouse.

MATERIALS AND METHODS

Untreated adult rats and mice were decapitated without anesthesia. The adrenals were quickly but carefully removed and dissected free of the surrounding fat.

The fluorescence method. Fresh-frozen sections were cut at 50 μ with a freezing microtome. The frozen sections were allowed to thaw on the surface of a solution containing 1 volume of commercial formalin, kept over calcium carbonate, 5 volumes of 2% calcium chloride, and 4 volumes of distilled water. After 2–6 hours the sections were rinsed in

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distilled water, floated on slides and mounted in glycerol. They were then examined under a fluorescence microscope.

The iodate method. Attempts to apply the iodate reaction to fresh-frozen sections were unsuccessful. The medulla became immediately red in the iodate solution but the colored substances, probably adrenochrome and noradrenochrome, soon dissolved in the reagent and no further color appeared. Only whole adrenals were therefore used subsequently. These were plunged into the iodate reagent immediately after removal. After 2–72 hours the glands were transferred into 10% formalin, where they were allowed to stay for 1–24 hours. Thereafter, frozen sections were cut at 50 μ. The sections were rinsed in distilled water and mounted in glycerol.

In the beginning, buffered solutions containing 2.5% of potassium iodate were used (Hillarp and Hökfelt, 1953). Without regard to the acidity or the kind of buffer used, the results were unsatisfactory, wherefore a saturated solution of potassium iodate was then adopted (Hillarp and Hökfelt, 1955).[1]

RESULTS

Earlier observations with the fluorescence method were confirmed. In the adrenal medulla of the rat, strongly fluorescing islets of cells were seen, the fluorescence being localized in the cytoplasm of these cells (Fig. 1). The adrenal medulla of the mouse behaved similarly (Fig. 3). However, the fluorescence of the islet cells was less intense in the mouse adrenal. Therefore, and because of the small size of the medullary cells in this species, the cytoplasmic localization of the fluorescence is not visible in the photograph, although there was no doubt about it upon visual examination. The medullary islets of the mouse were as a rule larger than those of the rat; in both species they seemed to be randomly distributed in the medulla.

As to the iodate reaction, immersion in saturated potassium iodate overnight and subsequent fixation in formalin for 2 hours yielded equally satisfactory results as longer periods of treatment (72 hours and 24 hours, respectively), which have been recommended by Hillarp and Hökfelt (1953, 1954 and 1955). Yellow-brown cell groups were demonstrated in the adrenal medulla of both species (Figs. 2 and 4). The intensity of the color produced was low and frequently variable even in different cells of each positive cell group. The fluorescence intensity, on the other hand, was always closely of the same order in all cells of the fluorescent islets demonstrated by the formalin treatment.

Although the weak and variable contrast rendered some difficulties in the study of the iodate-positive medullary islets, there was nevertheless little doubt that these islets were similar in shape, size and distribution to the fluorescent islets in the formalin-fixed sections from the contralateral adrenal of each animal, as can be seen by comparing Figures 1–4.

The intracellular distribution of the iodate reaction was different from that of the fluorescence in the islet cells. The whole cell was colored and, if there was any difference in the intensity of darkening between the nu-

[1] I am grateful to Dr. Hillarp who kindly suggested the use of a saturated solution in a letter to me in December 1954.

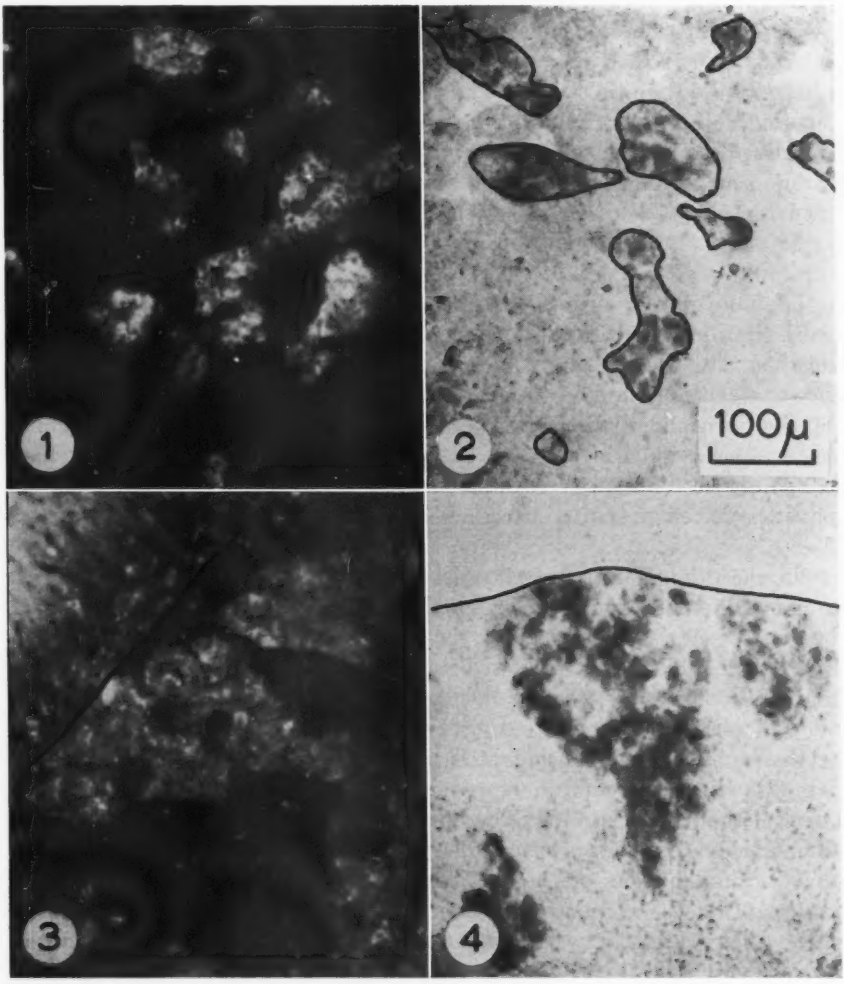

FIG. 1. Fluorescence photomicrograph of the adrenal medulla of a normal rat. Formalin fixation.

FIG. 2. Photomicrograph of the medulla in the contralateral adrenal of the same rat. Iodate-positive islets outlined with India ink. Iodate reaction.

FIG. 3. Fluorescence photomicrograph of the corticomedullary junction of a normal mouse. Cortex in the left upper corner. The junction is indicated by a black line. Formalin fixation.

FIG. 4. Photograph of the contralateral adrenal of the same mouse. Cortex above, medulla below; the corticomedullary junction is drawn black. Iodate reaction.

Magnification in all figures ×160.

cleus and the cytoplasm, the former seemed to be usually darker than the latter. Another difference between the results obtained with the two methods was that the iodate reaction was more intense in the medullary islets of the mouse than those of the rat, while the converse applied to the intensity of the formalin-induced fluorescence. The other two species hitherto examined, i.e. the cat and the hamster, show both an intense fluorescence after formalin fixation and an intense iodate reaction in the adrenomedullary islets.

DISCUSSION

The above observations further support the view suggested by previous work that both the fluorescence method and the iodate method selectively demonstrate the noradrenaline-containing cells of the adrenal medulla. Theoretically one might of course claim that the cell islets demonstrated by the former method are not necessarily identical with the islets stained brown by the iodate treatment, although the distribution, size, and shape of the respective islets were similar. In view of previous evidence (see the papers referred to in the introduction) such a possibility seems very unlikely.

On the other hand, one may ask whether both of these reactions are really due to noradrenaline and whether the intensity of either reaction can be regarded as an indicator of the relative noradrenaline concentration in these cells. These questions deserve serious consideration because the two methods gave discrepant results concerning the intensity of the reaction. If the fluorescence intensity of the medullary cells be a reliable guide to the noradrenaline concentration in these cells, fluorescent islets of the rat adrenal would contain more noradrenaline than the corresponding islets of the mouse adrenal. The opposite is suggested by the observation that the iodate reaction in medullary islets of the mouse is stronger than that in similar islets of the rat. The iodate reaction would furthermore indicate that the noradrenaline content of each individual cell in a single islet varies considerably in both rat and mouse adrenals, while the fluorescence reaction seemingly shows that all islet cells in each gland contain roughly equal concentrations of noradrenaline.

In vitro, mixing of pure noradrenaline and formalin produces a poorly water-soluble, fluorescent precipitate (Eränkö, 1955). A brown, poorly water-soluble precipitate is on the other hand obtained by mixing pure noradrenaline and potassium iodate (Hillarp and Hökfelt, 1953). This seems to show that noradrenaline is responsible for both reactions. However, it must be kept in mind that cells specialized in the production of noradrenaline may well differ in many respects from the cells producing adrenaline. Indeed, there is a sharp contrast in the acid phosphatase activities of these two types of cells in the rat (Eränkö, 1952). Therefore, also the fluorescence of the islet cells demonstrable after treatment with

formalin may be due to some substance other than noradrenaline. Still, all observations hitherto made, particularly the fact that nicotine injection, which causes a drop in the noradrenaline content of the medulla, abolishes the fluorescence of the islet cells, are compatible with the view that the fluorescence is directly caused by a reaction between formalin and noradrenaline. However, whether such a reaction can occur quickly enough to fix the fluorescent reaction product before noradrenaline has diffused out of the fresh-frozen section into the formalin solution, is questionable.

In all the species hitherto examined, the fluorescence has been observed exclusively in the cytoplasm of the islet cells. This agrees with studies of the intracellular distribution of adrenomedullary catechols by differential centrifugation (Blaschko and Welch, 1953; Hillarp, Lagerstedt and Nilson, 1953), which have unequivocally shown that these catechols are almost exclusively present in the cytoplasmic granules. Thus, the fluorescence —even if it is not directly due to noradrenaline—has a similar intracellular localization as noradrenaline. The iodate reaction, which is positive also in the nuclei, does on the other hand not truly indicate the distribution of noradrenaline in the cells—even if it is specifically due to this catechol amine. It seems likely that diffusion complicates the iodate method, as shown by the failure to give any permanent coloration in adrenal sections. Such diffusion might indeed explain the variations in the intensity of the iodate reaction observed in the present study. The smallest islets, i.e. those of the rat, give the weakest reaction; a stronger color is seen in the—somewhat larger—islets of mouse adrenals, and a strong reaction is observed in the—still larger—islets of cat and hamster adrenals.

In conclusion, it may be stated that the two histochemical methods are still incompletely known, wherefore more work is necessary to elucidate their significance. Although these methods cannot be recommended as substitutes for biological or chemical adrenaline and noradrenaline determinations, they may provide interesting information not obtainable with such determinations alone. Already at present it seems fairly well established that the two cell types demonstrable with them are independent of each other and that each of them is specialized in the secretion of one of the two adrenomedullary hormones.

SUMMARY

Two histochemical reactions, both discriminating between two different kinds of adrenomedullary cells, were applied to rat and mouse adrenals. One of the methods shows fluorescent cell islets in the medulla of formalin-treated adrenals against a non-fluorescent background and the other one brown islets against a yellow background in the medulla of iodate-treated adrenals. Distribution, size and shape of the cell groups demonstrable with the two methods are identical. It is believed that these cell groups contain and secrete noradrenaline. The fluorescence reaction is positive exclusively

in the cytoplasm, while the iodate reaction colors both the cytoplasm and the nuclei of the islet cells.

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QUANTITATIVE STUDY OF SPERMATOGENESIS IN THE HYPOPHYSECTOMIZED RAT

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THE rapid regression of the seminiferous tubules of the rat following hypophysectomy has been repeatedly observed since the original investigation of Smith (1930). This author as well as Crooke and Gilmour (1938), Tonutti (1943), and others reported that spermatids disappeared from the regressed tubules of hypophysectomized rats but that spermatogonia and spermatocytes as well as Sertoli cells persisted. Smith (1930) even noted the presence of spermatogonial mitoses months after hypophysectomy.

Except in the recent work of Mess (1952) no attempt had been made to assess quantitatively the precise changes taking place during spermatogenesis and resulting in a partial rather than total regression of seminiferous tubulues. In fact, until quite recently even the normal processes of spermatogenesis had not been investigated by means of quantitative techniques and the mode of proliferation and renewal of spermatogonia had remained obscure. The recent use of such techniques allowed a precise study of the evolution of the cellular population in the seminiferous epithelium (Roosen-Runge and Giesel, 1950; Leblond and Clermont, 1952b; Clermont and Leblond, 1953).

It was known that the cells present in the seminiferous tubules of the rat are arranged into well defined groups referred to as "cellular associations." With time, all the possible cellular associations occur successively in any given area of the tubules. Each sequence of a complete series of associations occurring in one area is known as a "cycle of the seminiferous epithelium." It was shown (Clermont and Leblond, 1953) that spermatocytes appear in the epithelium following 5 successive spermatogonial divisions, after the second of which one spermatogonium out of four stops dividing, while the others go through the last 3 divisions leading to spermatocyte formation. The non-dividing spermatogonium however resumes mitosis at the next cycle, when it acts as the stem cell which will again go through 5 successive divisions. The repetition of this pattern of spermatogonial divisions provides, cycle after cycle, new stem cells as well as successive generations of spermatocytes ("Stem Cell Renewal Theory" of Clermont and Leblond, 1953).

Following this study of spermatogenesis in the normal rat, it appeared of

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interest to apply the same histometric technique to an accurate investigation of spermatogenesis in the hypophysectomized rat. The quantitative data obtained allowed a precise description of the effects of pituitary extirpation on spermatogenesis.

METHODS

Twenty-five adult male albino rats were hypophysectomized (Charles River Breeding Laboratories) and sacrificed in groups of 3 at various time-intervals after operation, that is at 3, 6, 10, 15, 25, 29, 44 and 61 days. Normal controls were sacrificed as well throughout the course of the experiment. The testes were removed while the animals were under light ether anesthesia and fixed in Helly's fluid. Following paraffin embedding, five micra thick transverse sections were made from the middle portion of the testes. To permit identification of the various cell types and of the various stages of the cycle (according to the classification of Leblond and Clermont, 1952a), the sections were stained with the periodic acid-Schiff-hematoxylin technique.

Cell counts were made in sections from only one of two animals per group. The sections of testis to be analyzed quantitatively were photographed at low magnification (×30) and large prints made. Each cross-section of seminiferous tubules was located under the microscope. The stage of the cycle was then identified and recorded on the photograph of that tubule. The number of mitoses present was similarly noted. The number of Sertoli nuclei, type A and B spermatogonia, spermatocytes and spermatids was recorded in those cross-sections of tubules which happened to be at stages V and VII of the cycle. Twenty cross-sections of each of these two stages of the cycle were analyzed per animal.

RESULTS

Histology of the seminiferous epithelium of the rat after hypophysectomy

By 3 days after hypophysectomy, some of the maturing *spermatids* (steps 17–19)[1] were phagocytosed by the Sertoli elements instead of being released in the lumen. They were seen undergoing degeneration in the Sertoli cytoplasm close to the basement membrane. This process of degeneration was even more prominent at 6 days (Fig. 4) after which time all the maturing spermatids disappeared from the seminiferous epithelium.

At 6 days, degeneration also affected spermatids at earlier periods of spermiogenesis such as steps 9 and 10, that is, when the nucleus of the spermatids started elongating to transform into the sickle shaped head of the spermatozoon. At 10 days most of the spermatids reaching step 9 disintegrated and at 15 days after hypophysectomy no spermatids reached a later stage of development. At that time, however, young spermatids at steps 1 to 7 were still numerous. (In fact, small numbers of these spermatids were seen at all time intervals after hypophysectomy.)

At 6 days after hypophysectomy a few pachytene *spermatocytes* degenerated by pycnosis and at later time intervals the number of degenerating figures increased progressively. However, primary and secondary

[1] According to the terminology of Leblond and Clermont (1952a, b) the spermiogenesis of the rat was subdivided into a series of 19 stages or steps.

spermatocytes never disappeared completely from the seminiferous epithelium.

Ten days after hypophysectomy the nuclei of the *Sertoli elements* started to shrink but they did not undergo disintegration. Their cytoplasm became vacuolated.

While degeneration was observed in spermatids and spermatocytes no clearcut sign of degeneration was visible in the various types of *spermatogonia* (type A, intermediate type, and type B). Up to the latest period available (61 days after operation) normal mitoses of these cells were seen (see mitosis at 29 days in Fig. 7) and resting spermatocytes continued to be produced.

By 29 days after hypophysectomy the seminiferous epithelium had reached what appeared to be its maximum state of regression, since thereafter the histological picture did not change. The small seminiferous tubules contained, along a thickened basement membrane, 2 or 3 rows of cells recognized as type A, intermediate type and type B spermatogonia, primary and secondary spermatocytes, step 1–7 spermatids, and Sertoli elements with their contracted nuclei.

The stages of the cycle of the seminiferous epithelium and their frequency in hypophysectomized rats

In normal adult rats, the cells of the seminiferous epithelium form well defined cellular associations which, in any one place of the tubules, follow each other in time to constitute the "cycle of the seminiferous epithelium." It had been shown further (Leblond and Clermont, 1952a) that the frequency with which the various stages of the cycle appeared in cross sections of testes was characteristic of each stage and varied little from animal to animal. In hypophysectomized rats the identification of the stages of the cycle became increasingly difficult since the progressive disappearance of maturing spermatids from the seminiferous epithelium removed the best landmarks for the identification of the stages of the cycle in the classification of Leblond and Clermont (1952a). However, a careful examination of the other types of cells remaining in the seminiferous tubules, including young spermatids, permitted the identification of some of the stages, since cellular associations comparable to those described in the normal were observed. The frequency of the stages (expressed in percentages) which could be identified according to the criteria used in the normal were estimated up to 25 days after hypophysectomy.

Examination of these data (Table 1) showed that at the various time intervals after hypophysectomy the frequency of occurrence of most of the stages of the cycle (stages IV, VI, VII–VIII, IX, XII–XIII and XIV) was rather similar to that observed in the normal. The differences in the frequency of occurrence of the other stages may not be a real one, since these were the stages which were the most difficult to identify in regressing

TABLE 1. PERCENTAGES OF THE VARIOUS STAGES OF THE CYCLE AT VARIOUS TIME INTERVALS AFTER HYPOPHYSECTOMY

Stage of the cycle	Number of days after hypophysectomy					
	Normal	3	6	10	15	25
I	11.7	17.0	20.6	18.5	11.7	21.0
II–III	8.7	10.6	3.7	4.5	1.8	2.6
IV	3.9	3.6	2.8	2.5	4.7	3.9
V	8.5	5.8	6.9	7.8	4.5	3.5
VI	4.8	3.4	3.8	5.6	5.8	3.5
VII–VIII	24.9	24.7	27.9	28.4	35.3	20.6
IX	5.6	6.8	6.8	10.7	7.7	
X	6.6	2.0	2.9	3.6	11.2	41.6
XI	5.4	2.5	2.1			
XII–XIII	16.4	19.3	18.4	14.3	13.5	
XIV	3.3	4.3	3.8	3.8	4.1	3.2
Number of tubules counted	1000	632	674	552	444	310

seminiferous tubules. Thus it was felt that up to 25 days after hypophysectomy and presumably thereafter the relative frequency of the stages of the cycle was not much different from that in the normal.

Mess (1952) investigating the frequency of various types of seminiferous tubules in the hypophysectomized rat, but using a different classification of the stages of the cycle, could no longer differentiate the various stages of the cycle at and after 20 days following hypophysectomy.

Counts of cells at various time intervals after hypophysectomy

Sertoli cells. In the normal testis, the large, ill-defined cytoplasm of Sertoli elements contained an ovoid or triangular nucleus with a prominent nucleolus, two heterochromatic granules, and fine granular chromatin. After hypophysectomy, the cytoplasm became vacuolated and accumulated cellular debris. Simultaneously the nucleus decreased in size (from an average of 9.3 in the normal to 6.0 micra 61 days after operation); the nuclear membrane became wrinkled and the chromatin appeared denser. The nucleolus also was reduced in volume and became difficult to recognize.

Enumeration of Sertoli nuclei (Table 2, crude counts) gave a figure of 12.5 nuclei per cross section of tubule up to 3 days after hypophysectomy, 22.0 at 10 and 15 days, and 33.5 at 29 days, with no significant variation thereafter.

Spermatogonia. At stages V and VII of the cycle, the number of *type A spermatogonia* per tubular cross section (Table 2, crude counts) averaged 1.6 in normal rats as well as in hypophysectomized animals 3 and 6 days after operation. Subsequently this number increased slightly to reach 2.6, 3.2 and 2.4 cells at 29, 44, and 61 days respectively.

The number of *type B spermatogonia* present at stage V of the cycle de-

TABLE 2. NUMBER OF CELLS PER CROSS SECTION OF SEMINIFEROUS TUBULE* AT VARIOUS TIME INTERVALS AFTER HYPOPHYSECTOMY

Number of days after hypophysectomy	Type A spermatogonia		Type B spermatogonia		Resting spermatocyte		Pachytene spermatocyte		Spermatids (Step 1–7)		Sertoli cells		Sertoli‡ correcting factor
	Crude counts	True† counts	Crude counts	True counts	Crude counts	True counts	Crude counts	True counts	Crude counts	True counts	Crude counts	True counts	
normal	1.6	0.7	21.2	9.6	38.1	19.8	50.0	19.7	132.0	68.0	12.5	4.4	1.0
3	1.6	0.7	19.4	8.8	40.7	21.2	42.0	16.5	129.3	65.6	12.5	4.5	1.0
6	1.5	0.6	16.6	7.5	35.3	18.4	36.8	14.5	114.6	58.0	14.2	5.2	0.8
10	2.1	0.9	24.0	10.9	47.3	24.6	27.5	10.8	84.3	43.4	21.9	8.3	0.5
15	1.8	0.8	22.8	10.4	46.3	24.1	22.9	9.0	67.5	34.8	22.0	8.3	0.5
25	1.7	0.8	14.8	6.7	37.1	19.3	24.0	9.4	22.4	11.5	26.7	11.2	0.4
29	2.6	1.2	17.1	7.8	36.3	18.9	16.6	6.5	6.3	3.2	33.5	14.4	0.3
44	3.2	1.4	18.0	8.2	33.7	17.5	7.4	2.9	9.4	4.8	29.6	13.6	0.3
61	2.4	1.1	17.4	7.9	36.0	18.7	6.8	2.7	4.1	2.1	31.3	14.1	0.3

* Tubules at stage V and VII of the cycle.
† Crude counts corrected by Abercrombie formula.
‡ See explanation in text.

creased slightly after hypophysectomy (Table 2, crude counts). An average of 21.2 cells per tubular cross section was counted in the normal control and showed little change up to 15 days after hypophysectomy. At 25 days, and thereafter, the number of type B cells decreased and was found to be 17.4 at 61 days after operation.

No obvious signs of degeneration were found in the various types of spermatogonia and furthermore numerous mitoses of these were still observed 61 days after hypophysectomy.

Spermatocytes

The *resting spermatocytes* (present at stage VII of the cycle) were numerous in seminiferous tubules at all time intervals after pituitary extirpation (Table 2, crude counts). An average of 38.1 resting spermatocytes was found in the normal rat; their number increased slightly at 10 and 15 days after hypophysectomy. After 25 days this number remained around 36.0 per tubular cross-section. Some of these cells were seen undergoing degeneration.

Pachytene spermatocytes were counted at stages V and VII of the cycle (Table 2, crude counts). Their number which was 50.0 per tubular cross section in the normal rat steadily decreased after hypophysectomy to reach an average of 6.8 at 61 days. The marked reduction of the number of this type of cell can readily be correlated with the numerous images of degenerating spermatocytes seen in the seminiferous tubules.

Spermatids

In the normal rat, the number of spermatids at steps 1–7 of development averaged 132 per tubular cross section (Table 2, crude counts). This number rapidly decreased in the testes of hypophysectomized rats and reached 4.1, 61 days after operation. As already noted, step 8–19 spermatids had completely disappeared from the seminiferous tubules 15 days after hypophysectomy.

Correction of crude counts

Since the various cell types had different nuclear diameters, a correction of the crude counts had to be made before the analysis of the data could be undertaken. This correction was achieved, for all types of cells counted, by Abercrombie's formula,[2] $\left[T = C \times \frac{S}{S+d}\right]$ and the "true counts" obtained are given in Table 2.

Another factor had to be considered in order to make comparisons of counts from one animal to another, namely the progressive reduction in size of the seminiferous tubules after hypophysectomy. In addition to the decrease in diameter a reduction in length also took place, the latter being difficult to estimate from histological sections. This reduction in length of the tubules could be estimated tentatively from the counts of Sertoli nuclei. It was pointed out above that the number of Sertoli nuclei per tubular cross section increased markedly at various time intervals after hypophysectomy (Table 2, true counts). Since no mitosis of Sertoli cells was seen in the control nor in the experimental animals, the increase in number of these nuclei could not be due to cell divisions. Conversely, no degeneration of Sertoli nuclei had been observed either and it was, therefore, assumed that all those present in the normal were retained after hypophysectomy. Under these conditions, it was felt that the increase of the number of Sertoli nuclei could be explained by a shortening of the tubules, as a result of which more nuclei would appear per tubular cross section. Consequently the ratio of the true number of Sertoli nuclei counted per tubular cross section in the normal rat (Table 2, true counts) divided by the corresponding number at any given time interval after hypophysectomy was taken as an index of the reduction in length of the seminiferous tubules. Such indexes or Sertoli correcting factors (Table 2, last column) were used to correct the counts of the various types of nuclei and thus an attempt was made to eliminate the influence of the shortening of the seminiferous tubules. To do so, the true counts of spermatogonia, spermatocytes and spermatids (Table 2) were multiplied by these Sertoli correcting factors. The figures obtained or "corrected counts" are given in Table 3.

Observations on "corrected counts" of cells in the seminiferous epithelium of the rat after hypophysectomy (Table 3)

The number of *type A spermatogonia* which was 0.7 on the average in the normal rat, gradually decreased to reach 0.4 to 0.3 between 15 and 61 days after hypophysectomy (Table 3, Fig. 1). This was in contrast with the apparent increase in the number of type A cells observed from the crude counts (Table 2).

[2] T = true count, C = crude count, S = thickness of the section, d = diameter of the nucleus. For each cell type the diameter d was estimated from measurements made on 40 nuclei.

The number of *type B spermatogonia* showed a more pronounced decrease than was apparent from the crude counts. Their number, 9.5 in the normal, decreased to 2.7, 25 days after hypophysectomy and thereafter remained close to this low value (Table 3, Fig. 1).

The crude counts of *resting spermatocytes* at various time intervals after hypophysectomy had been found to be almost equal to the one estimated in normal rats. On the contrary the corrected counts showed that the actual

TABLE 3. NUMBER OF CELLS PER CROSS SECTION OF SEMINIFEROUS TUBULE AT VARIOUS TIME INTERVALS AFTER HYPOPHYSECTOMY

Corrected counts

Number of days after hypophysectomy	Type A spermato-gonia	Type B spermato-gonia	Resting spermato-cyte	Pachytene spermato-cyte	Spermatids
normal	0.7	9.6	19.8	19.7	68.0
3	0.7	8.8	21.2	16.5	65.6
6	0.5	6.0	14.7	11.6	46.4
10	0.5	5.5	12.3	5.4	21.7
15	0.4	5.2	12.0	4.5	17.4
25	0.3	2.7	7.7	3.8	4.6
29	0.4	2.3	5.7	2.0	1.0
44	0.4	2.5	5.2	0.9	1.4
61	0.3	2.4	5.6	0.8	0.6

number of cells decreased markedly in hypophysectomized animals. 19.8 resting spermatocytes were counted in the normal animals while only 5.7 to 5.2 were found 29 days after hypophysectomy and later (Table 2, Fig. 1).

The crude numbers of *pachytene spermatocytes* and of *step 1–7 spermatids* were found to decrease considerably after hypophysectomy. An even more pronounced drop was observed from the "corrected counts." The number of pachytene spermatocytes per tubular cross section fell from 19.7 in the normal of 0.8 by 61 days after hypophysectomy. The spermatid number which averaged 68.0 in the normal decreased to 0.6 by 61 days (Table 3, Fig. 1).

DISCUSSION

The cycle of the seminiferous epithelium in hypophysectomized rats

The first cells to disintegrate and disappear from the seminiferous tubules after hypophysectomy were the step 9–19 spermatids. The loss of these cells deeply changed the histological appearance of the seminiferous epithelium since the elongated nuclei of these spermatids which in the normal rats formed typical bundles inserted in the Sertoli elements were no longer present. The disappearance of these cells rendered difficult the identification of some of the stages of the cycle according to the classification of Leblond and Clermont (1952a) in which step 9–14 spermatids were used to identify the corresponding stages IX to XIV. Even so it was clear that the cycle of the seminiferous epithelium was maintained after hy-

pophysectomy. The remaining spermatogonia, spermatocytes and spermatids, even if they were reduced in number, were still arranged in typical and constant cellular associations which, with time, evolved into one another. The spermatogonia thus continued to proliferate rhythmically and to produce groups of isogenic spermatocytes which evolved synchronously

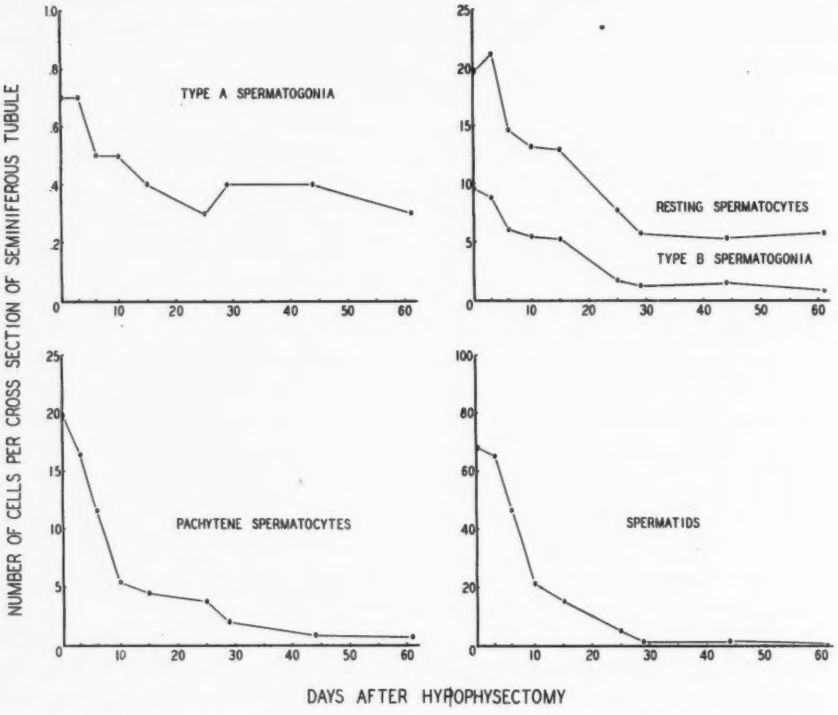


FIG. 1. Graphic representation of the corrected cell counts per tubular cross section (Table 3) at various time intervals after hypophysectomy. For all the germ cells counted (type A and B spermatogonia, resting and pachytene spermatocytes, step 1–7 spermatids) a progressive reduction in number was observed up to 25 days after hypophysectomy. After 25 days the number remained approximately constant.

at the same pace as in the normal. Furthermore up to 25 days after hypophysectomy, the frequency of the stage of the cycle (which was taken as an index of the relative duration of the stages of the cycle; Leblond and Clermont, 1952a) was found to be closely similar to that observed under normal conditions. This result showed that the timing of the evolution of the various types of cells persists after hypophysectomy and is in fact an essential character of the seminiferous epithelium.

Degeneration taking place during the proliferation of spermatogonia

The mode of proliferation and renewal of spermatogonia in normal adult rats has been described in detail by Clermont and Leblond (1953). At the

beginning of spermatogenesis, the type A stem cell divides twice to give 4 type A spermatogonia (Fig. 2). One of these stops dividing and is in fact a new type A stem cell; the other 3 type A cells divide and give rise to a new generation of cells called intermediate spermatogonia. These in turn divide to produce 12 type B spermatogonia which, in a last wave of divisions, produce 24 spermatocytes. The ratio of type A stem cell to type B cells in such a scheme thus is 1:12, while the ratio of type B cells to resting spermatocytes is 1:2.

Using the corrected counts of Table 3, the ratios of type A to type B cells as well as of type B cells to resting spermatocytes were calculated for the various time intervals after hypophysectomy (Table 4). These data allowed a precise estimation of the degeneration taking place during the proliferation of spermatogonia.

The ratio of type A to type B spermatogonia, theoretically 1:12 in the normal rat, varied between 1:11 and 1:13 up to 15 days after operation. At 25 days and later the ratios changed and varied between 1:9 and 1:6 (Table 4). Consequently the number of type B cells produced per type A stem cell remained comparable to normal up to 15 days after hypophysectomy, but thereafter the yield of type B cells per type A stem cell was subnormal. It has already been noted that a marked reduction of the number

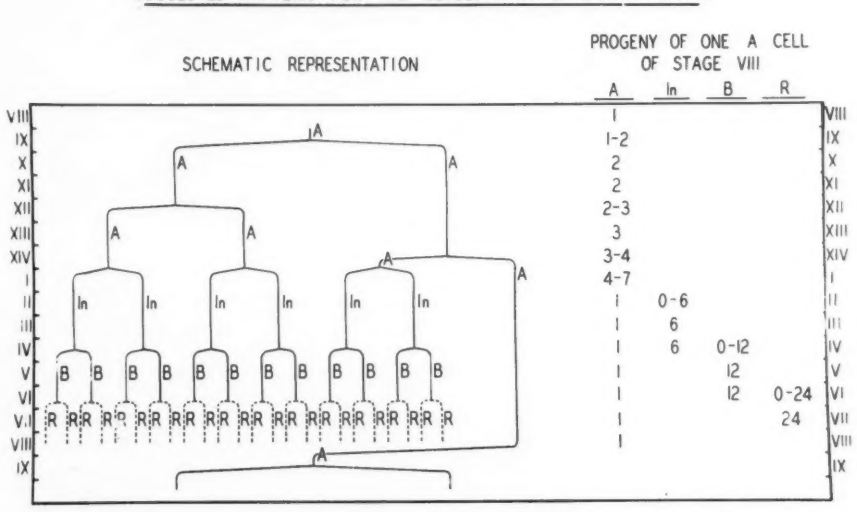
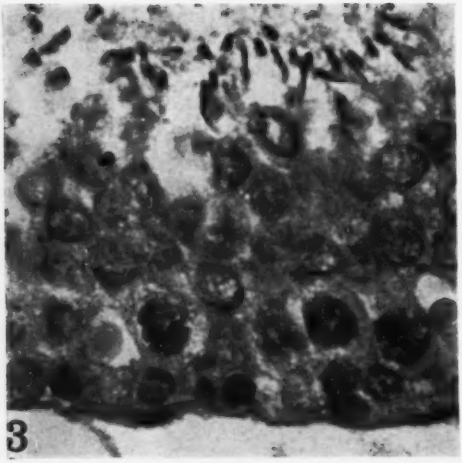


FIG. 2. Diagrammatic representation of the most probable pattern for the development and the mode of renewal of spermatogonia (or "Stem Cell Renewal Theory"). The Roman numerals on either side of the diagram indicate the stages of the cycle. Lettering: A, type A spermatogonia; In, intermediate type of spermatogonia; B, type B spermatogonia; R, resting spermatocytes. Each mitosis is indicated by the junction of a vertical and two horizontal lines. The Arabic numbers on the right hand side of the diagram are obtained by adding up the number of cells on the same horizontal lines. Taken from Clermont and Leblond ('53).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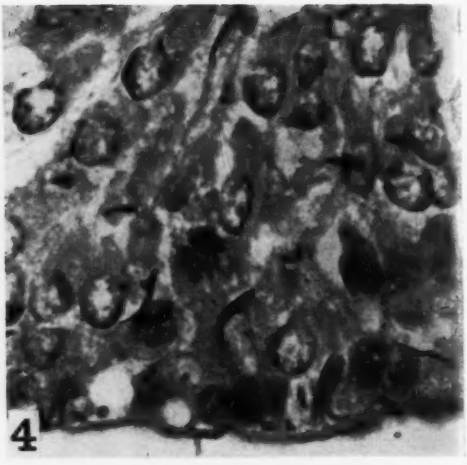
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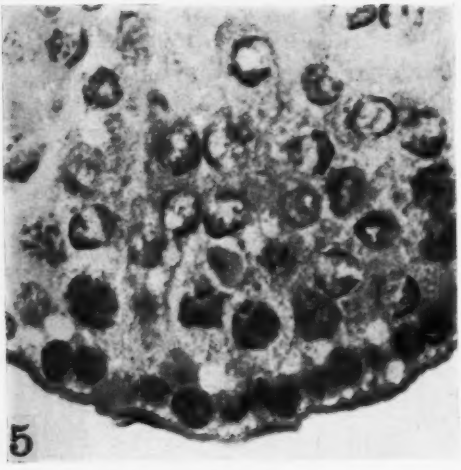
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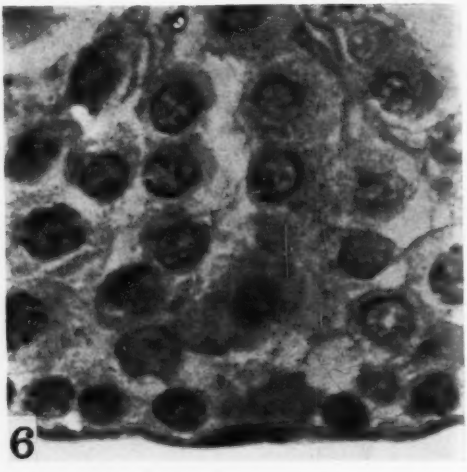
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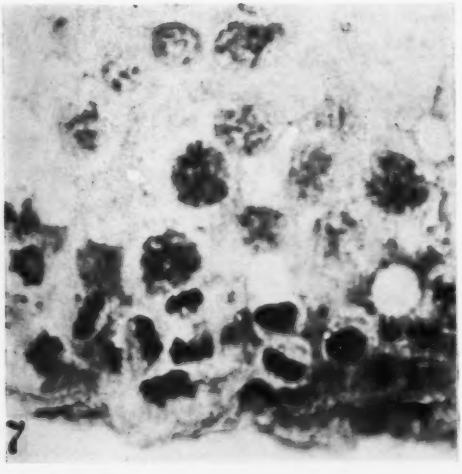
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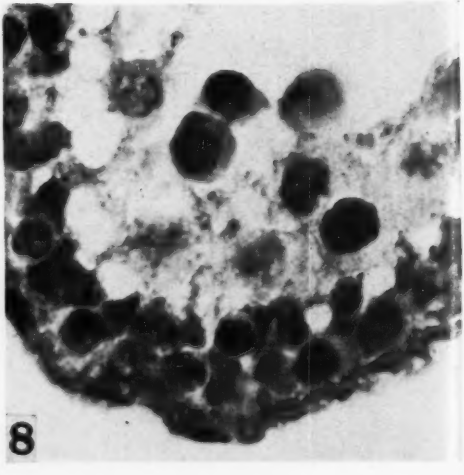
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of type A and B cells took place up to 15 days after hypophysectomy (Table 3, Fig. 1). To explain this reduction in view of the fact that during the same time intervals the yield of type B cell per type A stem cell was normal, a reduction of the number of type A stem cells should be postulated. At 25 days after hypophysectomy and later there was no further decrease of the number of spermatogonia (Table 3, Fig. 1). Since the num-

TABLE 4. RATIOS OF THE CELLS OF THE SEMINIFEROUS EPITHELIUM AT VARIOUS TIME INTERVALS AFTER HYPOPHYSECTOMY

Number of days after hypophysectomy	Type A sptg.[1] / Type B sptg.	Type B sptg. / Resting sptc.[2]	Resting sptc. / Pachytene sptc.	Pachytene sptc / Spermatids
normal	1:13	1:2.0	1 :1	1:3.5
3	1:12	1:2.4	1.3:1	1:4.0
6	1:12	1:2.4	1.3:1	1:4.0
10	1:11	1:2.2	2.3:1	1:4.0
15	1:13	1:2.3	2.6:1	1:3.9
25	1: 9	1:2.8	2.0:1	1:0.8
29	1: 6	1:2.4	2.9:1	1:2.0
44	1: 6	1:2.0	5.8:1	1:0.7
61	1: 8	1:2.3	6.2:1	1:1.2

[1] Sptg.: spermatogonia.
[2] Sptc.: spermatocytes.

ber of type B cells produced per type A stem cell was subnormal, degeneration of some spermatogonia must also have taken place up to the fourth spermatogonial mitoses.

PLATE 1

EXPLANATION OF FIGURES

Cross sections of seminiferous tubules at various time intervals after hypophysectomy. All tubular sections are at stage VIII of the cycle except in fig. 7 which is at stage VI of the cycle. Fixation in Helly's fluid. Stained by the periodic acid-Schiff technique and counterstained with hematoxylin. Magnification approximately ×900.

FIG. 3. Three days after hypophysectomy. Resting and pachytene spermatocytes, step 8 spermatids with their head caps oriented toward the basement membrane and spermatozoa lining the lumen of the tubule can be seen.

FIG. 4. Six days after hypophysectomy. The spermatozoa instead of being released normally into the tubular lumen appeared to be phagocytized by the Sertoli elements and therefore are close to the basement membrane.

FIG. 5. Fifteen days after hypophysectomy. The spermatozoa have disappeared from the seminiferous tubule. Although many step 8 spermatids are present, no spermatids at more advanced steps of spermiogenesis are visible.

FIG. 6. Twenty-five days after hypophysectomy. All cell types except spermatozoa are present.

FIG. 7. Twenty-nine days after hypophysectomy. Numerous mitoses are still present along the basement membrane. In this instance the dividing cells are type B spermatogonia.

FIG. 8. Sixty-one days after hypophysectomy. Type A spermatogonia, resting spermatocytes and contracted Sertoli nuclei formed the basal layer of cells along the basement membrane; pachytene spermatocytes and spermatids lie in a foamy cytoplasmic substance close to the lumen of the tubule.

The ratios of type B cells to resting spermatocytes (Table 4) remained close to the theoretical ratio 1:2 at all time intervals after hypophysectomy (Table 4). This indicated that whenever type B spermatogonia were produced, they divided normally into resting spermatocytes. Thus, mitoses of type B spermatogonia were not affected by the lack of hypophyseal hormone.

The above mentioned ratios as well as the abundance of mitoses clearly indicated that spermatogenesis was not completely arrested. In fact, spermatogonial proliferation proceeded actively and spermatocyte production continued, although at a reduced rate, at all time intervals after hypophysectomy.

Degeneration taking place during meiosis

The marked reduction in the number of spermatocytes (Fig. 1) in hypophysectomized rats was obviously due to a degeneration of some type A spermatogonia and also to the pronounced degeneration of the spermatocytes themselves during the long meiotic prophase. The degeneration of spermatocytes during meiosis, which was easily observed as early as 10 days after hypophysectomy, had already been reported by previous investigators, but no attempt had been made to assess it quantitatively. Such an evaluation was attempted by examining at each time interval after hypophysectomy the ratios of resting spermatocytes to pachytene spermatocytes and of pachytene spermatocytes to spermatids, which should, theoretically, be 1:1 and 1:4 respectively (Table 4).

The ratio of resting spermatocytes to pachytene spermatocytes (Table 4) progressively changed and became close to 6:1, 44 and 61 days after hypophysectomy. This indicated that at these time intervals, of 6 resting spermatocytes, only one reached the pachytene stage of meiosis at stage VII of the cycle, the other 5 having degenerated during the leptotene, zygotene and early pachytene stages of meiosis.

The ratio of pachytene spermatocytes to step 1–7 spermatids remained close to the expected value of 1:4, up to 15 days after hypophysectomy (Table 4). Thereafter the ratios varied between 1:2.0 and 1:0.7. Thus at 25 days and after, approximately one spermatid instead of four was produced per spermatocyte, indicating that 75% of the spermatocytes degenerated during the late prophase and the two maturation divisions of meiosis. From these various counts of cells it was estimated that approximately 4% of resting spermatocytes produced spermatids at 44 and 61 days after hypophysectomy.

Degeneration taking place during spermiogenesis

The marked reduction in the number of step 1–7 spermatids after hypophysectomy (Table 3, Fig. 1) was mostly due to degeneration taking place during earlier steps of spermatogenesis. Up to 61 days after hypophysec-

tomy step 1–7 spermatids were still being produced by the seminiferous epithelium of the rat. However, all the spermatids degenerated as they reached step 8 or 9 of spermiogenesis. Smith (1930) and Crooke and Gilmour (1938) did not mention the persistence of spermatids after hypophysectomy. Tonutti (1943) wrote that spermatids disappeared from the seminiferous tubules a few days after hypophysectomy. Mess (1952) observed that following hypophysectomy in the rat, the more differentiated were the first to degenerate but after 20 days all of them were gone. Moricard and Gothié (1939) observed spermatids three to four weeks after operation in some of their animals but attributed their presence to remaining pituitary tissue. The quantitative data obtained in the present series of hypophysectomized rats demonstrated that young spermatids are always present, although in small number, in some cross sections of seminiferous tubules, i.e., those which are at stages I–VII of the cycle.

Cutuly and Cutuly (1940) studying the endocrine control of the seminiferous epithelium divided spermatogenesis into three phases, a premeiotic, a reduction division phase, and a post-meiotic phase. According to these authors "the first phase seems to occur independently of any known hormonal stimulation, the second seems to be controlled by hypophyseal gonadotropin and the third is either independent of any recognized hormonal stimulation or else it progresses under the influence of testicular hormone." From the present quantitative study it appeared that spermatogenesis proceeds as far as step 7 of sperminogenesis in absence of the hypophyseal hormone(s). The presence of the pituitary gland was necessary however to permit the completion of spermiogenesis and also to maintain numerically the cellular population of the seminiferous epithelium to the normal level.

SUMMARY

The cell population of the seminiferous epithelium was analysed quantitatively in the testes of rats sacrificed up to 61 days after hypophysectomy. Although extensive degeneration took place after this operation, many cell types persisted in the seminiferous epithelium and remained grouped into cellular associations, the development of which evolved cyclically as in the normal rat. Counts of type A and B spermatogonia, resting and pachytene spermatocytes and young spermatids were obtained at stages V and VII of the cycle and corrected for the reduction in length of the seminiferous tubules.

The analysis of the data shows that the numbers of the cells of the seminiferous epithelium decrease markedly up to 25 days after hypophysectomy and remain approximately constant thereafter.

A partial degeneration of type A spermatogonia takes place during their period of proliferation. The type B spermatogonia divide into resting spermatocytes without loss. Marked degeneration of spermatocytes occurs

before and during meiosis in such a way that, at 44 and 61 days after hypophysectomy, only 4% of the spermatocytes appearing in the testis have produced spermatids. These spermatids evolve up to step 7 of spermiogenesis and then degenerate.

Thus, in the rat, spermatogenesis may proceed independently of hypophyseal hormone stimulation up to step 7 of spermiogenesis, but only a very small number of cells reach this stage.

Acknowledgments

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EFFECT OF PITUITARY EXTRACTS UPON THE WORK PERFORMANCE OF ADRENALECTOMIZED-HYPOPHYSECTOMIZED RATS: IDENTIFICATION OF VASOPRESSIN AS A PRINCIPLE AFFECTING WORK[1]

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THE rat loses its capacity to work in response to faradic stimulation within a few hours following either adrenalectomy or hypophysectomy. Adrenalectomized rats will work normally when given continuous intravenous injections of adrenal cortical extract (ACE) whereas the performance of hypophysectomized and adrenalectomized-hypophysectomized rats is improved but not restored to normal by ACE. The performance of either hypophysectomized or adrenalectomized-hypophysectomized rats is restored to normal by the continuous intravenous injection of ACE plus certain preparations of corticotropin (Ingle, Nezamis and Morley, 1953; Ingle *et al.*, 1953).

The data of the present study show that various fractions of pituitary extracts contain this principle affecting work; that a fraction containing this principle is separable from corticotropin; this principle is not identical with any known anterior lobe hormone; and finally that minute amounts of vasopressin added to ACE will normalize the work of adrenalectomized-hypophysectomized rats in acute experiments.

METHODS

Male rats of the Sprague-Dawley strain were obtained from the Upjohn breeding colony and maintained on Archer Dog Pellets until they reached a weight of 200 ± 2 gm. The work test was according to Ingle (1944). The animals were anesthetized with phenobarbital sodium and cyclopal sodium and were subjected to the stimulation of muscle immediately following hypophysectomy or adrenalectomy-hypophysectomy. The gastrocnemius muscle of the left hind leg was weighted with 100 gm. A Nerve Stimulator, Model B (Upjohn) was used to deliver 5 pulses per second. The duration of each pulse was 20 milliseconds and the intensity was 20 milliamperes. The distance the weight was lifted was recorded on automatic work adders. Each recorder revolution represented approximately 400 gram-centimeters of work. A set of modified recorders was used

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temporarily which gave somewhat lower values for work. Stimulation was continued until death or for 48 hours. Temperature was constant at 28 ± 0.5° C.

Preparation L 2397 BP was obtained from a partial peptic digest of ACTH fraction (Li *et al*, 1955) by the following procedure: one gram of fraction F and 12.5 mg. of crystalline pepsin were dissolved in 100 ml. 0.01 N HCl and kept at 37° for 3 hours. An equal volume of 50% trichloro-acetic acid (TCA) was added to the whole digest; after removal of the supernatant by centrifugation, the precipitate was dissolved in acid solu-

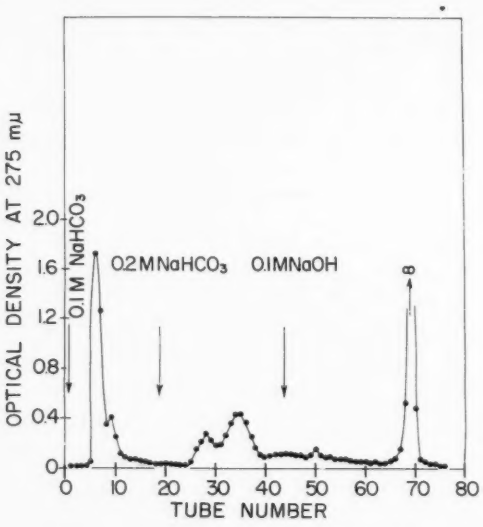

FIG. 1. Chromatography on the Na form of Amberlite IRC-50 resin (column 2.4 × 25 cm.) of an ACTH fraction (L2397BP, 189 mg. of fraction K); 20 ml. per tube.

tion, extracted with ether, then passed through an IR-4B column to remove the remaining TCA, and finally lyophilized.

Preparation KA was obtained by submitting L 2397 BP onto an XE-97 column using stepwise elution with 0.1 M $NaHCO_3$, 0.2 M $NaHCO_3$ and 0.1 M NaOH as eluents. A typical pattern may be seen in Figure 1. The material in tubes 5–10, 25–40 and 65–72 were combined, isolated and designated as fractions KA, KB and KD respectively.

Preparations CCIA, CCIB, and CCID were subfractions from KA after countercurrent distribution through 100 transfers using 0.5% TCA/2-butanol system in an all glass apparatus (Craig and Post, 1949). As shown in Figure 2, the material was collected in Tubes 6–11, 12–17, 18–44 and 45–80, and designated as CCIA, CCIB, CCIC and CCID respectively.

Growth hormone (L 2349 A) was prepared from anterior lobes of bovine pituitaries by the simplified procedure (Li, 1954). Lactogenic hormone was prepared from sheep glands by the method of Cole and Li (1955). Follicle-stimulating hormone and interstitial cell stimulating hormone were prepared from sheep glands (Li, Simpson and Evans, 1940, 1949). Intermedin was prepared from swine posterior pituitaries (Geschwind and Li, unpublished). Thyrotropin was a commercial product (Parke, Davis).

The preparation of corticotropin A (Armour) has been described by White and Fierce (1953) and corticotropin B (Merck) has been described by Brink, *et al.* (1952). Payne, Raben and Astwood (1950) have described the adsorption of the corticotropin

principle by cellulose. Taylor and du Vigneaud (1953) have reported on purified vasopressin.

The potency of corticotropin is expressed in terms of U.S.P. units as determined by the adrenal ascorbic acid method (Sayers, Sayers and Woodbury, 1948).

Each pituitary extract was added to physiological saline together with penicillin G sodium (5000 units per 20 ml.) and ACE. The ACE represented the activity equivalent of 0.1 mg. of hydrocortisone (1 unit) per ml. as determined by the liver glycogen deposi-

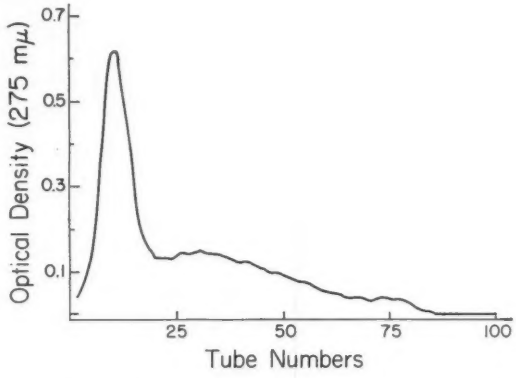

FIG. 2. Counter-current distribution (100 transfers) of material (85 mg. of fraction KA) obtained from chromatography on IRC-50 resin (material in tubes 5–10; see Figure 1). System, 2-butanol-0.5% trichloroacetic acid.

tion test (Pabst, Sheppard and Kuizenga, 1947). In the earlier experiments each rat received 20 units of ACE during each 24 hour period; in later experiments the dose was decreased to 10 units during the second 24 hours. An attempt was made to use less ACE but vascular collapse rapidly ensued in many of the rats during the first few hours following adrenalectomy-hypophysectomy. A volume of 20 ml. per 24 hours was injected into the jugular vein of each rat by means of a continuous injection machine which permitted the study of 6 rats simultaneously.

EXPERIMENTS AND RESULTS

Within each of the 31 experimental groups comparisons of replacement therapy were made simultaneously. The numbers of animals used were determined in part by amounts of the test substance available to us and by the objective of the particular experiment, i.e., whether it represented a pilot experiment or was intended to yield a definitive answer at this point of the study. The details of each experiment are given in the tables.

Experiment 1 (Table 1) involved 10 groups each of which represented two or more experimental conditions. It was confirmed that ACE alone supports the capacity of the adrenalectomized rat to work much more effectively than it supports work performance in the adrenalectomized-hypophysectomized rat (Group 1). A dissociation is shown between the potency of various preparations of corticotropin in causing the depletion of adrenal ascorbic acid and potency in supporting work under these condi-

TABLE 1. EFFECT OF ACE* AND SOME PREPARATIONS OF CORTICOTROPIN UPON MUSCLE WORK IN THE RAT; AVERAGES AND STANDARD ERRORS

Group	Experimental condition	Number rats	Treatment		U.S.P. units/mg.	Total average recorder revolutions
			Preparation	Mcg. ACTH/24 hrs. per rat		
1†	Adrenalectomy only	23	ACE only	0	—	93,103±2,040‡
	Adrenalectomy-Hypophysectomy	15	ACE only	0	—	61,410±3,910
	Adrenalectomy-Hypophysectomy	15	ACE plus corticotropin B (Merck)	10	57.0	60,789±3,380
	Adrenalectomy-Hypophysectomy	11	ACE plus 2397 BP (Li)	10	30.0	90,777±4,540‡
2†	Adrenalectomy-Hypophysectomy	19	ACE only	0	—	63,915±3,050
	Adrenalectomy-Hypophysectomy	19	ACE plus corticotropin B (Merck)	20	57.0	72,575±3,660
	Adrenalectomy-Hypophysectomy	10	ACE plus 2397 BP (Li)	2	30.0	79,625±4,030
3†	Adrenalectomy-Hypophysectomy	12	ACE only	0	—	64,118±5,980
	Adrenalectomy-Hypophysectomy	16	ACE plus corticotropin B (Merck)	50	57.0	72,943±3,790
4	Adrenalectomy-Hypophysectomy	11	ACE only	0	—	74,454±5,390
	Adrenalectomy-Hypophysectomy	8	ACE plus corticotropin A (Armour)	2	43.2	68,982±5,750
	Adrenalectomy-Hypophysectomy	12	ACE plus corticotropin A (Armour)	10	43.2	71,347±3,570
5	Adrenalectomy-Hypophysectomy	11	ACE only	0	—	63,747±4,040
	Adrenalectomy-Hypophysectomy	9	ACE plus corticotropin A (Armour)	25	43.2	61,576±3,400
6	Adrenalectomy-Hypophysectomy	19	ACE only	0	—	78,029±4,370
	Adrenalectomy-Hypophysectomy	19	ACE plus crude corticotropin (Astwood)	1,000	0.6	105,041±4,320‡
7	Adrenalectomy-Hypophysectomy	15	ACE only	0	—	76,667±4,580
	Adrenalectomy-Hypophysectomy	14	ACE plus unabsorbed fraction (Astwood)	1,000	0.05	88,949±3,180
	Adrenalectomy-Hypophysectomy	15	ACE plus unabsorbed fraction (Astwood)	2,500	0.05	92,144±3,790‡
8	Hypophysectomy only	18	ACE only	0	—	69,729±4,245
	Hypophysectomy only	10	ACE plus absorbed fraction (Astwood)	100	22.7	74,888±3,660
	Hypophysectomy only	13	ACE plus absorbed fraction (Astwood)	250	22.7	74,513±6,880
	Hypophysectomy only	10	ACE plus absorbed fraction (Astwood)	500	22.7	82,172±6,520
	Hypophysectomy only	10	ACE plus corticotropin (Upjohn)	500	22.7	94,640±4,930‡
9	Adrenalectomy-Hypophysectomy	19	Saline only	0	—	29,667±1,320‡
	Adrenalectomy-Hypophysectomy	19	ACE only	0	—	71,345±3,930
	Adrenalectomy-Hypophysectomy	12	ACE plus absorbed fraction (Astwood)	100	22.7	69,517±6,130
	Adrenalectomy-Hypophysectomy	19	ACE plus absorbed fraction (Astwood)	500	22.7	80,369±3,760
10	Adrenalectomy-Hypophysectomy	13	ACE only	0	—	76,720±5,910
	Adrenalectomy-Hypophysectomy	8	ACE plus purified ACTH (Li)	2	43.7	67,399±5,470
	Adrenalectomy-Hypophysectomy	12	ACE plus purified ACTH (Li)	25	43.7	71,592±7,020

* ACE was administered in the amount of 20 units per rat during each 24 hour period.
† Temporary recorders.
‡ Statistical probability is 99 per cent or greater that a true difference exists between this average and the average for the adrenalectomized-hypophysectomized rats of this group treated with ACE only.

tions. The most highly purified preparations of corticotropin had little or no effect on work (Groups 2, 3, 4, 5, 8, 9 and 10).

Experiment 2 (Table 2) involved 7 groups representing highly purified pituitary hormones. Although large doses of growth hormone (Group 11)

TABLE 2. WORK PERFORMANCE OF ADRENALECTOMIZED-HYPOPHYSECTOMIZED RATS TREATED WITH ACE* AND HIGHLY PURIFIED PITUITARY HORMONES; AVERAGES AND STANDARD ERRORS

Group	Experimental condition	Number rats	Treatment		Total average recorder revolutions
			Preparation	Mcg./24 hrs. per rat	
11†	Adrenalectomy-Hypophysectomy	24	ACE only		*62,500*±3,140
	Adrenalectomy-Hypophysectomy	12	ACE plus growth hormone L2349A(Li)	250	52,679±5,830
	Adrenalectomy-Hypophysectomy	12	ACE plus growth hormone L2349A (Li)	1,000	83,408±5,030‡
12	Adrenalectomy-Hypophysectomy	11	ACE only		68,648±6,710
	Adrenalectomy-Hypophysectomy	11	ACE plus lactogenic hormone L2378B (Li)	100	72,821±3,020
13†	Adrenalectomy-Hypophysectomy	12	ACE only		59,378±4,200
	Adrenalectomy-Hypophysectomy	12	ACE plus lactogenic hormone L2378B (Li)	500	61,882±4,780
14	Adrenalectomy-Hypophysectomy	10	ACE only		72,345±6,750
	Adrenalectomy-Hypophysectomy	8	ACE plus TSH (Li)	25	65,487±6,920
	Adrenalectomy-Hypophysectomy	8	ACE plus FSH (Li)	25	62,455±6,790
15	Adrenalectomy-Hypophysectomy	8	ACE only		78,874±8,610
	Adrenalectomy-Hypophysectomy	8	ACE plus ICSH (Li)	25	78,706±6,290
16†	Adrenalectomy-Hypophysectomy	9	ACE only		57,054±4,480
	Adrenalectomy-Hypophysectomy	8	ACE plus intermedin R527109)Armour)	2	43,634±6,490
	Adrenalectomy-Hypophysectomy	8	ACE plus intermedin R527109 (Armour)	25	51,575±6,680
17	Adrenalectomy-Hypophysectomy	14	ACE only		77,718±5,020
	Adrenalectomy-Hypophysectomy	16	ACE plus intermedin (Li)	2	80,291±5,340
	Adrenalectomy-Hypophysectomy	12	ACE plus intermedin (Li)	25	72,497±5,390

* ACE was administered in the amount of 20 units per rat during each 24 hour period.
† Temporary recorders.
‡ Statistical probability is 99 per cent or greater that a true difference exists between this average and the average for the adrenalectomized-hypophysectomized rats of this group treated with ACE only.

TABLE 3. SEPARATION OF FRACTION AFFECTING WORK FROM ADRENAL ASCORBIC ACID DEPLETING ACTIVITY; AVERAGES AND STANDARD ERRORS. ACE DOSAGE 20 UNITS PER RAT DURING EACH 24 HOUR PERIOD

Group	Experimental condition	Number rats	Treatment			Total average recorder revolutions
			Preparation*	Mcg./24 hrs. per rat	U.S.P. units/mg.	
18†	Sham-Operated	15	Saline only			81,390±3,132‡
	Adrenalectomy-Hypophysectomy	15	ACE only			*52,091*±6,372
19†	Adrenalectomy-Hypophysectomy	17	ACE only			*51,588*±3,760
	Adrenalectomy-Hypophysectomy	15	ACE plus fraction "A"	0.5	0.64	65,564±3,000‡
	Adrenalectomy-Hypophysectomy	15	ACE plus fraction "A"	2.0	0.64	87,000±2,620‡
	Adrenalectomy-Hypophysectomy	15	ACE plus fraction "A"	10.0	0.64	93,552±3,920‡
20†	Adrenalectomy-Hypophysectomy	12	ACE only			*58,965*±3,740
	Adrenalectomy-Hypophysectomy	12	ACE plus fraction "B"	2.0	50.0	60,109±3,680
	Adrenalectomy-Hypophysectomy	12	ACE plus fraction "B"	10.0	50.0	58,068±3,590
	Adrenalectomy-Hypophysectomy	12	ACE plus fraction "B"	25.0	50.0	57,800±3,950
21†	Adrenalectomy-Hypophysectomy	8	ACE only			*55,127*±4,960
	Adrenalectomy-Hypophysectomy	8	ACE plus fraction "D"	2.0	3.0	57,092±6,410
	Adrenalectomy-Hypophysectomy	8	ACE plus fraction "D"	10.0	3.0	56,621±7,740
	Adrenalectomy-Hypophysectomy	8	ACE plus fraction "D"	25.0	3.0	47,358±4,300
22†	Adrenalectomy-Hypophysectomy	8	ACE only			50,142±6,760
	Adrenalectomy-Hypophysectomy	7	ACE plus fraction "35A"	2.0	Not Tested	60,330±5,430
	Adrenalectomy-Hypophysectomy	8	ACE plus fraction "35A"	10.0		59,995±4,370
	Adrenalectomy-Hypophysectomy	8	ACE plus fraction "35A"	25.0		54,234±5,150
23†	Adrenalectomy-Hypophysectomy	12	ACE only			*63,208*±6,210
	Adrenalectomy-Hypophysectomy	12	ACE plus fraction "A" (new prep)	2.0	0.5	102,326±4,250‡

* All fractions separated from 2397 BP (Li).
† Temporary recorders.
‡ Statistical probability is 99 per cent or greater that a true difference exists between this average and the average for the adrenalectomized-hypophysectomized rats of this group treated with ACE only.

had a positive effect on work none of these pituitary principles supported work in doses comparable to the effective doses of partially purified corticotropin 2397 BP. It was therefore concluded that the extra-adrenal effect of corticotropin 2398 BP is not due to contamination with any of the pituitary principles tested in Experiment 2.

Experiment 3 (Table 3) involved 6 groups representing 4 fractions derived from partially purified corticotropin 2397 BP (see METHODS). The ascorbic acid depleting activity was concentrated in fraction B and the

TABLE 4. SEPARATION OF FRACTION AFFECTING WORK FROM INTERMEDIN. AVERAGES AND STANDARD ERRORS. ACE DOSAGE 20 UNITS PER RAT DURING 1ST 24 HOUR PERIOD AND 10 UNITS DURING SECOND 24 HOUR PERIOD

Group	Experimental condition	Number rats	Treatment		Total average recorder revolutions
			Preparation*	Mcg./24 hrs. per rat	
24	Adrenalectomy-Hypophysectomy	6	ACE plus fraction KA	50	93,016±4,379†
	Adrenalectomy-Hypophysectomy	15	ACE only		*43,206*±4,833
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIA	10	47,464
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIA	30	37,504
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIA	50	46,638
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIA	100	68,310
25	Adrenalectomy-Hypophysectomy	15	ACE only		*49,999*±6,836
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIB	10	32,807
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIB	30	36,058
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIB	50	48,701
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIB	100	43,068
26	Adrenalectomy-Hypophysectomy	6	ACE plus fraction KA	2	94,453±6,517
	Adrenalectomy-Hypophysectomy	9	ACE only		*51,306*±7,604†
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIC	10	102,442
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIC	4	98,649
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCIC	1	61,072
27	Adrenalectomy-Hypophysectomy	6	ACE plus fraction KA	1	83,970±6,795†
	Adrenalectomy-Hypophysectomy	6	ACE plus fraction KA	2	87,707±2,850†
	Adrenalectomy-Hypophysectomy	12	ACE only		*52,640*±6,410
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCID	30	103,646
	Adrenalectomy-Hypophysectomy	3	ACE plus fraction CCID	10	80,475
	Adrenalectomy-Hypophysectomy	6	ACE plus fraction CCID	4	64,893±7,188

* Fractions CCIA, B, C, and D were separated from fraction KA which is identical with fraction A of Table 3. Some intermedin activity was found in fractions CCIA, CCIB and CCIC; fraction CCID was free from intermedin and adrenal ascorbic acid depleting activity.
† Statistical probability is 99 per cent or greater that a true difference exists between this average and the average for the adrenalectomized-hypophysectomized rats of this group treated with ACE only.

factor affecting work was concentrated in fraction A thereby supporting the conclusion that the two principles are not identical.

Experiment 4 (Table 4) involved 4 fractions derived from fraction A (now called KA) of Experiment 3. Since fraction A was found to contain melanophore stimulating activity as tested in hypophysectomized frogs (Reinhardt, *et al.*, 1952) further fractionation was carried out by counter current distribution (see METHODS). Fractions CCIC and CCID affected work but the potency was less than in the mother fraction KA. Fraction CCID was free of melanophore stimulating activity thereby adding to the evidence summarized in Table 2 (Groups 16 and 17) that the extra-adrenal principle affecting work is not identical with intermedin.

TABLE 5. EFFECT OF POSTERIOR PITUITARY EXTRACTS UPON WORK. AVERAGES AND STANDARD ERRORS. ACE DOSAGE 20 UNITS DURING FIRST 24 HOUR PERIOD AND 10 UNITS DURING SECOND 24 HOUR PERIOD

Group	Experimental condition	Number rats	Treatment		Total average recorder revolutions
			Preparation	Units/24 hrs. per rat	
28	Adrenalectomy-Hypophysectomy	15	ACE only		*36,097*± 4,312
	Adrenalectomy-Hypophysectomy	7	ACE plus pitressin (Parke, Davis)	0.002	53,591± 7,846
29	Adrenalectomy-Hypophysectomy	31	ACE only		*36,151*± 2,919
	Adrenalectomy-Hypophysectomy	7	ACE plus pitressin (Parke, Davis)	0.005	72,288± 6,112*
	Adrenalectomy-Hypophysectomy	6	ACE plus pitressin (Parke, Davis)	0.01	80,244± 3,367*
	Adrenalectomy-Hypophysectomy	7	ACE plus pitressin (Parke, Davis)	0.02	93,937± 5,901*
	Adrenalectomy-Hypophysectomy	7	ACE plus pitressin (Parke, Davis)	0.05	75,235± 4,361*
	Adrenalectomy-Hypophysectomy	8	ACE plus pitressin (Parke, Davis)	0.1	68,381± 6,088*
30	Adrenalectomy-Hypophysectomy	20	ACE only		*42,237*± 4,073
	Adrenalectomy-Hypophysectomy	8	ACE plus pitocin (Parke, Davis)	0.0025	37,829± 5,910
	Adrenalectomy-Hypophysectomy	8	ACE plus pitocin (Parke, Davis)	0.005	36,220± 5,284
	Adrenalectomy-Hypophysectomy	8	ACE plus pitocin (Parke, Davis)	0.01	43,993± 6,169
	Adrenalectomy-Hypophysectomy	8	ACE plus pitocin (Parke, Davis)	0.025	44,068± 8,656
	Adrenalectomy-Hypophysectomy	8	ACE plus pitocin (Parke, Davis)	0.05	40,870± 8,680
31	Adrenalectomy-Hypophysectomy	25	ACE only		*42,130*± 3,623
	Adrenalectomy-Hypophysectomy	16	ACE plus vasopressin (du Vigneaud)	0.005	72,898± 4,913*
	Adrenalectomy-Hypophysectomy	16	ACE plus vasopressin (du Vigneaud)	0.01	90,420± 4,646*
	Adrenalectomy-Hypophysectomy	16	ACE plus vasopressin (du Vigneaud)	0.02	93,885± 2,576*

* Statistical probability is 99 per cent or greater that a true difference exists between this average and the average for the adrenalectomized-hypophysectomized rats of this group treated with ACE only.

During tests of fraction KA, it was noted that doses having an optimal effect in enhancing work also inhibited the diuresis which began within two hours after hypophysectomy in animals given ACE only. We therefore re-examined the possibility that the extra-adrenal effect on work could be due to the presence of vasopressin. A sample of fraction KA was submitted to Professor H. B. van Dyke, Department of Pharmacology, Columbia University College of Physicians and Surgeons, who estimated its pressor action in rats and its antidiuretic effect in hydrated dogs. It was found to represent the activity equivalent of 2 units of U.S.P. standard per mg.

Experiment 5 (Table 5) included tests of a commercial preparation of vasopressin (Parke, Davis, Pitressin), found to be efficacious with as little as 0.005 U.S.P. units per rat per 24 hours; a commercial preparation of oxytocic principle (Parke, Davis, Pitocin), found ineffective at comparable dosage; and a pure preparation of vasopressin supplied by Professor V. du Vigneaud of Cornell University Medical College, found to be efficacious in doses as small as 0.005 units per rat per 24 hours. The dose of 0.005

units did not prevent diuresis; the dose of 0.01 units delayed the onset of diuresis in some of the animals; and a dose of 0.02 units, optimal in its effect on work, inhibited diuresis in all of the animals.

DISCUSSION

The conclusion that vasopressin is probably the principle in certain anterior pituitary extracts having an extra-adrenal effect on work is based upon the following considerations: 1. It was found possible to separate this principle from partially purified preparations of corticotropin. This fraction (KA) did not represent the activity of any of the known anterior lobe principles. It was possible to separate intermedin from fraction KA. 2. Fraction KA was found to contain a significant amount of pressor and antidiuretic activity. 3. Pure vasopressin added to ACE has been found to normalize the work performance of adrenalectomized-hypophysectomized rats under the conditions of these experiments. 4. Purified preparations of the other known pituitary hormones fail to have this effect.

It would now be in order to attempt the production of a chronic insufficiency of vasopressin and to study the effect of insufficiency and of replacement therapy upon work. This has not been done.

It would appear from the data of Experiment 5, Group 29 (Table 5) that doses higher than 0.02 units per 24 hours per rat have a suboptimal effect on work, i.e. overdosage.

Many experiments were conducted before definitive tests were done on vasopressin. It is of interest to review why this was so. 1. Unpublished tests of a commercial preparation of vasopressin (Parke, Davis, Pitressin) were carried out more than 3 years ago. It is now apparent that those rats were overdosed some thousandfold and that the dose-response effect was not adequately explored. 2. The preparations of corticotropin used in these initial studies of an extra-adrenal effect on work were described as practically free from posterior pituitary principles. The biologic significance of small quantities of the pressor principle was not appreciated by us. 3. Early experiments led us to conclude that there was no effect of leaving the posterior lobe *in situ* during hypophysectomy whereas incomplete removal of the anterior lobe was thought to afford some protection against loss of vigor. These observations were of the cursory sort. Too little attention was paid to whether or not the pituitary stalk was left intact and whether or not diuresis occurred following partial hypophysectomy. 4. Most important of all, our psychological bias favored the anterior lobe of the pituitary as the source of the extra-adrenal principle affecting work.

SUMMARY

Male rats of 200 gm. were anesthetized with barbiturates and following operation were subjected to faradic stimulation of the gastrocnemius muscle to lift a 100 gm. weight 5 times per second. Stimulation was con-

tinued until the death of the rat or for 48 hours. The following operations were represented: sham, adrenalectomy, hypophysectomy and adrenalectomy-hypophysectomy. Hormones were administered by continuous intravenous injection. In confirmation of earlier studies it was found that certain pituitary extracts contain a principle which has an extra-adrenal effect upon the work performance of the hypophysectomized rat. The principle was shown to be separable from corticotropin and to differ from each of the known anterior lobe hormones and from oxytocin and intermedin. It was found that minute amounts of vasopressin added to adrenal cortical extract will normalize the work of adrenalectomized-hypophysectomized rats under the conditions of these experiments. Vasopressin was found to be present in anterior pituitary extracts which supported work.

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The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

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In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. In 1955 the Medal was presented to Dr. Carl R. Moore.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945, Dr. Jane A. Russell; 1946, Dr. Martin M. Hoffman; 1947, Dr. Choh Hao Li; 1948, Dr. Carl Heller; 1949, Dr. George Sayers; 1950, Dr. Oscar M. Hechter; 1951, Dr. Albert Segaloff; 1952, Dr. Seymour Lieberman; 1953, Dr. Sidney Roberts and Dr. Claire Szego (Mrs. Roberts); 1954, Dr. Isodore M. Rosenberg; 1955, Dr. Jack Gross. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-fourth months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

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This fellowship was established in 1947. It is designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. The fellowship is awarded on alternate years (1955, 1957, etc.) and the stipend which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree or candidates for either of these degrees are eligible for appointment.

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(2) Recommendations from individuals familiar with the candidate and his work.
(3) A proposed program of study.
(4) Acceptance of the individual by the head of the department in which the Fellowship will be held.
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The award will not exceed $2,500 annually for each individual, and shall be granted on the basis of proposals submitted by the applicant and such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

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Nominations for the Medal of The Endocrine Society; the Ciba Award; and the Ayerst, McKenna and Harrison Fellowship may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 N. Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than November 1st each year.

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